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Methods in Plant Histology

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METHODS IN PLANT HISTOLOGY

METHODS

IN

PLANT HISTOLOGY

BY

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CHICAGO

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PREFACE.

This book has grown out of a course in histological technique conducted by the author at the University of Chicago. The course has also been taken by non-resident students through the Extension Division of the University. The METHODS were published over a year ago as a series of articles in the *Journal of Applied Microscopy*, and have called out numerous letters of commendation, criticism, suggestion, and inquiry. The work has been thoroughly revised and enlarged by about one-half. It is hoped that the criticism and suggestion, and also the experience gained by contact with both resident and non-resident students, have made the directions so definite that they may be followed, not only by those who work in a class under the supervision of an instructor, but also by those who must work in their own homes without any such assistance.

More space has been devoted to the paraffin method than to any other, because it has proved to be better adapted to the needs of the botanist. The celloidin method, the glycerine method, and free-hand sectioning are also described, and their advantages and disadvantages are pointed out.

The first part of the book deals with the principles of fixing and staining, and the various other processes of microtechnique, while in the later chapters these principles are applied to specific cases. This occasions some repetition, but the mere presentation of general principles will not enable the beginner to make good mounts.

The illustrations and notes in the later chapters are not intended to afford a study of general morphology, but they

merely indicate to students with a limited knowledge of plant structures the principal features which the preparations should show. The photomicrographs were made from the author's preparations by Dr. W. H. Knap, and *figs. 52*, *57*, and *59* were drawn by Miss Eleanor Tarrant; all other figures of plant structures were made from the author's drawings.

Corrections and suggestions will be heartily appreciated.

CHARLES J. CHAMBERLAIN.

CHICAGO,
June 1, 1901.

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Part I.

CHAPTER I.

APPARATUS.

The following list of apparatus includes a fair equipment for histological work: a microscope magnifying at least 400 diameters; a hand microtome; a sliding microtome; a razor; a hone and a good razor strop; a paraffin bath and lamp; a turn-table; a scalpel; a pair of needles; a pair of scissors; a pair of forceps; stender dishes; minots or watch-glasses; a wash bottle; a graduate (50 or 100 cc.); pipettes; slides, 1×3 inches; round covers, 18 mm. or ¾ inch in diameter; and square covers, ⅞ inch. Long covers, 22×50 mm., will be needed for some of the serial sections.

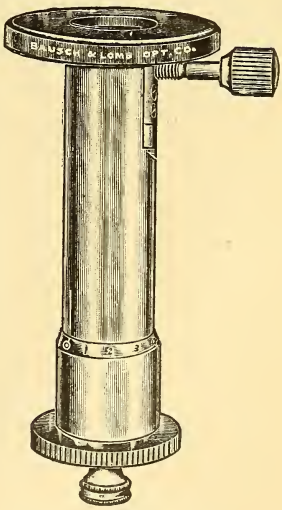

Fig. 2. A convenient form of hand microtome.

A convenient and effective microscope should have a rack and pinion coarse adjustment, a fine adjustment, two eyepieces (about one-inch and two-inch preferred), a low-power objective of two-thirds of an inch or a one-inch focus, a high-power objective of one-fifth or one-sixth of an inch focus, a double nosepiece, an iris diaphragm, and an Abbé condenser. A cheap and practical form is shown in *fig. 1*, and similar instruments are for sale by all the leading companies.

The hand microtome (*fig. 2*) will be found extremely useful, especially by the busy teacher who has large classes. Any sliding microtome, if kept in good order, will be sufficient for the work to be described in this book, but those of medium size are to be preferred. The student's microtome (*figs. 3* and *3 A*) is quite inexpensive and does good

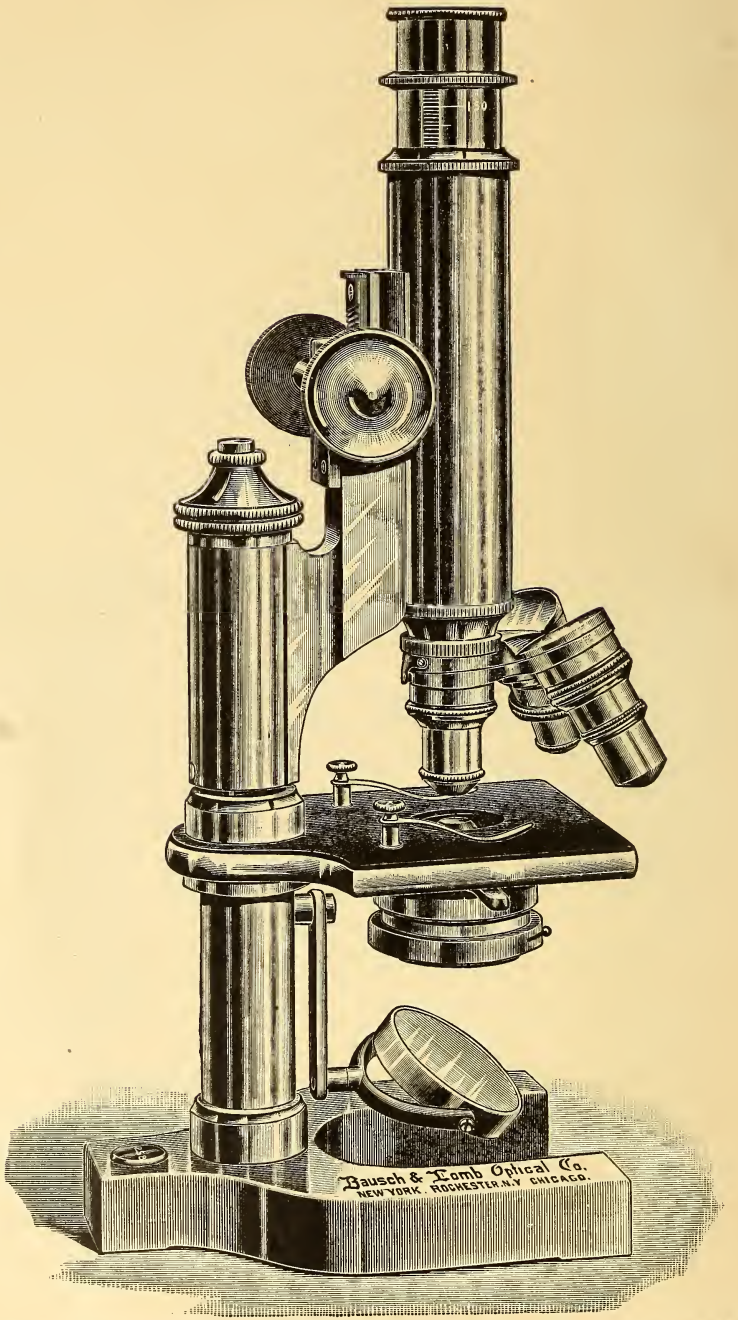

FIG. 1. A compound microscope, with rack and pinion coarse adjustment, micrometer screw fine adjustment, triple nosepiece, iris diaphragm, and condenser.

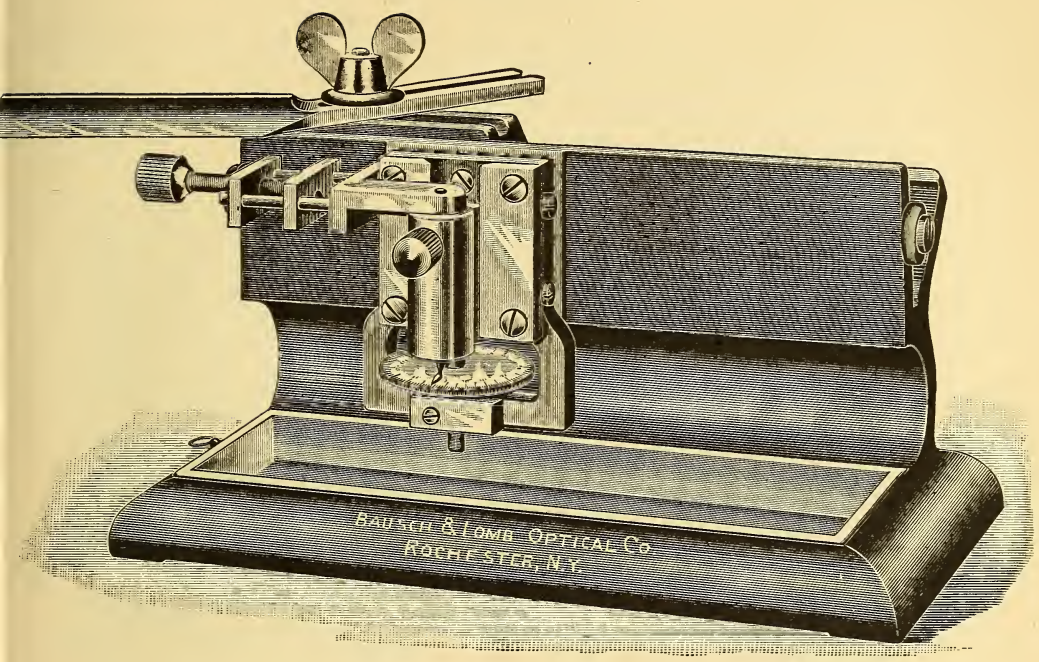

FIG. 3. The student's microtome.

work. Where expense is not an objection the Jung Thoma, the Minot, and other microtomes of similar grade are to be preferred.

The stout razors our grandfathers used to shave with are excellent for free-hand sectioning, for hand-microtome work, and even for cutting

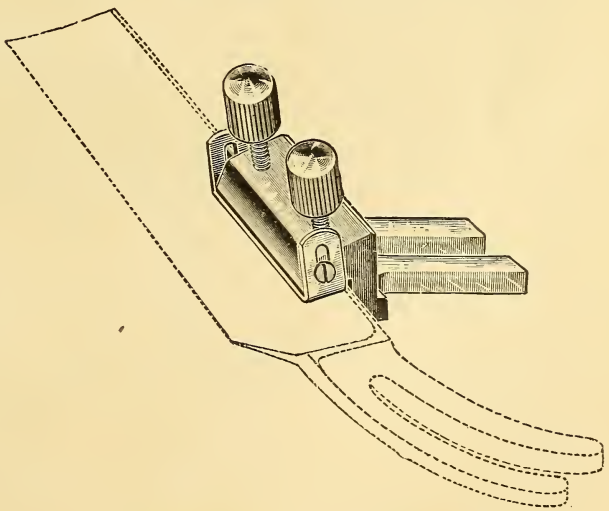

FIG. 3*A*. Clamp to hold an ordinary razor in the student's microtome.

paraffin sections on the sliding microtome. The blade should be flat on one side (*fig. 4 A*). Modern razors (*fig. 4 B*) with delicate blades, though good to shave with, are worthless for cutting sections of plants. The razor is a necessity; if a microtome knife is wanted in addition, it should have a bevel about like that shown in *fig. 4 A*. A short blade, two or three inches in length, is to be preferred to the longer ones, which are much more troublesome to sharpen.

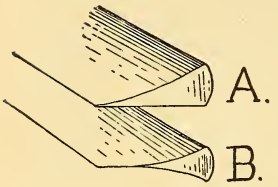

FIG. 4.

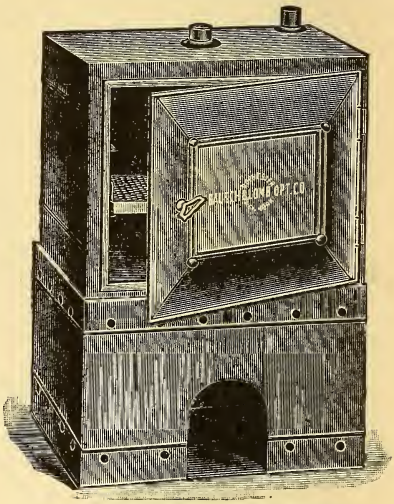
FIG. 5. A paraffin bath, with water-jacket, designed to be used with a thermostat.

There are numerous forms of the paraffin bath. Those with a water-jacket, a thermometer, and a thermostat to maintain an even temperature are the most convenient where gas is available (*fig. 5*). As a rule it is easier to keep the temperature constant in the larger baths. A bath which, if carefully watched, gives the very best results can be made by any tinner, and is very inexpensive. The accompanying figures show the form and dimensions (*fig. 6*).

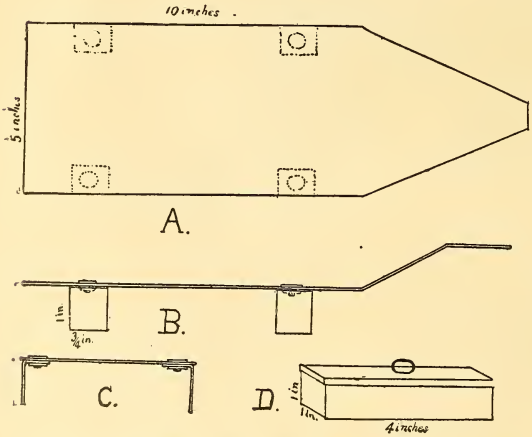

FIG. 6. *A*, top view; *B*, side view; *C*, end view; *D*, box to contain the paraffin.

It is made of copper one thirty-second

of an inch thick, but thicker copper is as good or better. There should be two boxes to contain the paraffin; the covers to the boxes should fit loosely. Any kind of a lamp may be used.

Stender dishes are now very generally used for staining on the slide. The form shown in *fig. 7 A* is made just large enough to hold two slides, placed back to back, and hence requires only a minimum of the reagent. The cap in this form does not fit closely enough to keep absolute alcohol and xylol, but does very well for the other alcohols and stains. We do not believe that the convex cover is as good as a flat one. The form shown in *fig. 7 B* is the best for absolute alcohol and xylol, but even with this it is better to put a little vaseline or glycerine on the cover to prevent any evaporation. Wide-mouthed bottles, though not so convenient, give just as good results.

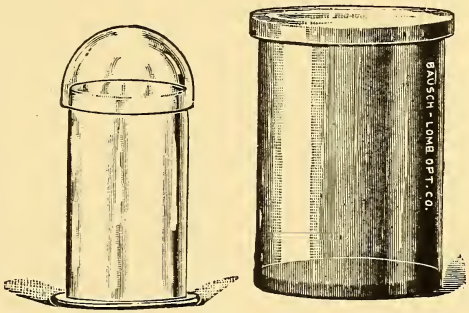

FIG. 7. *A*, Naples jar; *B*, stender dish.

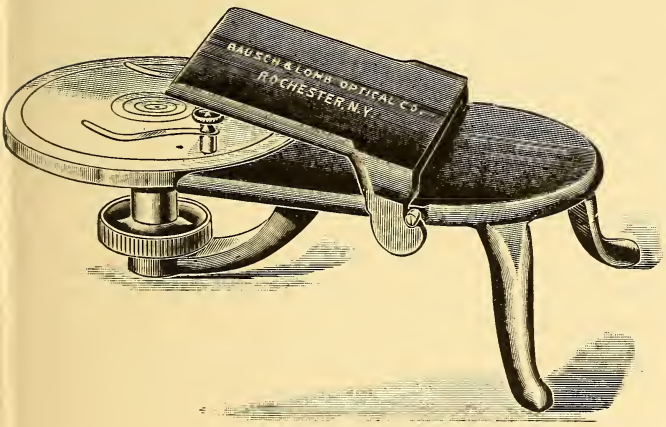

FIG. 8. Turn table.

A serviceable form of turn-table for glycerine mounts is shown in *fig. 8*.

The other pieces of apparatus mentioned need no comment. By consulting a catalogue, which will be furnished by any dealer, the beginner can determine what he needs to buy, and what he can find substitutes for, if it is necessary to be very economical.

CHAPTER II.

REAGENTS.

It would require entirely too much space even to enumerate the reagents which are occasionally used in a fully equipped university laboratory. The following list includes only those which are used constantly. *The Microtomist's Vade-Mecum* by Lee contains very complete formulæ for stains and other reagents. The quantities mentioned below indicate about what the average student uses in a three-months' course in methods. Nearly all the stains, however, would last for a year, if properly used.

KILLING AND FIXING AGENTS.

Commercial alcohol (about 95 per cent.), 2 liters; absolute alcohol, 300 cc.; ether, 50 cc.; chromic acid, 10 g.; corrosive sublimate, 10 g.; glacial acetic acid, 25 cc.; hydrochloric acid, 50 cc.; picric acid, 5 g.; chloroform, 50 cc.; [osmic acid, 1 per cent. solution in water, 25 cc. This is extremely expensive, and not necessary except for the most delicate work]. Formulæ for making killing and fixing agents from these materials will be given later.

STAINS.

Only a few of the most important stains are given in this list. In general one should have enough of a stain to stand about two inches high in the stender dish or bottle in which the staining is to be done. The theory and practice of staining will be discussed later.

Delafield's Hæmatoxylin.— To 100 cc. of a saturated solution of ammonia alum add, drop by drop, a solution of 1 g. hæmatoxylin dissolved in 6 cc. of absolute alcohol. Expose to air and light for one week, then filter. Add 25 cc. of glycerine and 25 cc. of methyl alcohol. Allow to stand until the color is rather dark. Filter, and keep in a tightly stoppered bottle. The solution

should stand for two months before it is ready for use, but, if needed immediately, the "ripening," which is brought about by the oxidation of hæmatoxylin into hæmatin, may be secured in a few minutes by a judicious addition of peroxide of hydrogen.

Mayer's Hæm-Alum.—Dissolve with gentle heat 1 g. of hæmatoxylin in 50 cc. of 95 per cent. alcohol; add a solution of 50 g. of alum in a liter of distilled water. Allow the mixture to cool and settle; filter; add a crystal of thymol to preserve from mold. The stain is ready for use as soon as made, and it keeps well.

Haidenhain's Iron Alum-Hæmatoxylin.—Two solutions are used, and they are never to be mixed:

(*a*) A 1½ to 4 per cent. aqueous solution of ammonia sulphate of iron.

(*b*) A ½ per cent. aqueous solution of hæmatoxylin.

Cyanin, Erythrosin, Safranin, Gentian Violet.—Numerous formulæ are given for these and other anilin stains, but the following general formula gives excellent results:

Make a 3 per cent. solution of anilin oil in distilled water; shake thoroughly and frequently for a day; add enough alcohol to make the whole mixture about 20 per cent. alcohol. Add 1 g. of cyanin or erythrosin, etc., as the case may be, to 100 cc. of the solution. Safranin is often used in a strong alcoholic solution, and even with the above formula it is better to dissolve the safranin in strong alcohol before adding it to the mixture.

Acid Fuchsin.—Use a 1 to 2 per cent. solution in water, or 70 per cent. alcohol.

Iodine Green.—Use a 1 to 4 per cent. solution in water or alcohol. A 3 per cent. solution in 70 per cent. alcohol is very good for the vascular system of plants.

Mixtures of Fuchsin and Iodine Green.—The following formula is often used for karyokinetic figures:

(*a*) A ½ per cent. solution of fuchsin in water.

(*b*) A ½ per cent. solution of iodine green in water.

Just before using mix (*a*) and (*b*) in various proportions until what is needed for the particular case is found.

Orange G.—Use a saturated aqueous solution.

Eosin.— A 1 to 5 per cent. solution in water or alcohol. A 2 per cent. aqueous solution is good for material to be mounted in glycerine, but a 2 per cent. solution in 70 per cent. alcohol is better for balsam mounts. The stronger solution may be diluted as needed for special cases.

FORMULÆ FOR ALCOHOLS.

The grades of alcohol in most common use are 35 per cent., 50 per cent., 70 per cent., 85 per cent., 95 per cent., and 100 per cent. The 100 per cent. is expensive, and great care should be taken to keep the bottle well corked or the stender dish closely covered. The following formulæ will enable anyone to make the other grades of alcohol from 95 per cent. alcohol and water:

95	35	95	50	95	70	95	85
—		—		—		—	
60		45		25		10	

The above are the formulæ for 35 per cent., 50 per cent., 70 per cent., and 85 per cent. alcohol. Any other grade can be gotten in the same way. In the first formula, subtract 35 from 95; the result, 60, is the number of cubic centimeters of water which must be added to 35 cc. of 95 per cent. alcohol in order to obtain 35 per cent. alcohol. The mixture contains 95 cc. of 35 per cent. alcohol. If more or less than 95 cc. of the mixture is needed, take proportional parts of 35 and 60. This simple method is a time-saver, but if the bottles or stender dishes are to be filled frequently, it will be a still further saving of time to use a long label (*fig. 9*), and, after pouring in the 95 per cent. alcohol, draw a line showing how high it reaches, and then, after pouring in the water, draw another line. The next time it is necessary to fill the bottles merely pour in 95 per cent. alcohol until it reaches the first line, and then pour in water until it reaches the second line. It is not necessary to use distilled water, if pure drinking water is available.

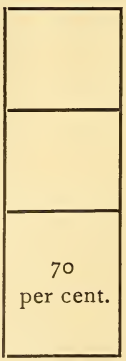

FIG. 9.

CLEARING AGENTS.

Xylol is the most generally useful clearing agent yet known. Clove oil, cedar oil, bergamot oil, carbolic acid, and turpentine are all necessary for special purposes. About 200 cc. of xylol, 50 cc. of clove oil, and 25 cc. of each of the others makes a fair outfit to begin with.

MISCELLANEOUS.

Canada balsam, 25 cc.; glycerine, 50 cc.; glycerine jelly, 25 cc.; 2 per cent. celloidin, 50 cc.; 10 per cent. celloidin, 50 cc.; hard and soft paraffin, 500 g. each; gold size, 25 cc.; and a small soft brush for ringing glycerine mounts.

ARRANGEMENT OF THE OUTFIT FOR STAINING AND MOUNTING.

It is best to keep the various reagents in definite positions in order that no time may be lost in hunting for anything. The

FIG. 10.

accompanying diagram (*fig. 10*) of a part of the top of a table shows a convenient arrangement.

The alcohols are in front and the stains are placed behind. The eosin, fuchsin, iodine green, cedar oil, and clove oil may be kept in bottles; the rest should be in stender dishes.

CHAPTER III.

TEMPORARY MOUNTS.

Before considering the complicated methods involved in making permanent preparations a word should be said about temporary mounts. A preliminary examination of almost any botanical material may be made without any fixing, imbedding, or staining. If a little starch be scraped from a potato, and a small drop of water and a cover-glass be added, a very good view will be obtained, and if a small drop of iodine solution be allowed to run under the cover, the preparation, while it lasts, could hardly be improved. The unicellular and filamentous algæ can be studied quite satisfactorily from such mounts. The protonema of mosses and the prothallia of ferns should be studied in this way, even if a later study from sections is intended. If the top of a moss capsule be cut off at the level of the annulus, a beautiful view of the peristome may be obtained by simply mounting in a drop of water, or, in a case like this where no collapse is to be anticipated, the object may be mounted in a small drop of glycerine—just enough to come to the edge of the cover without oozing out beyond—and the preparation made permanent by sealing with gold size or any good cement. The antheridia and archegonia of mosses may be examined if the surrounding leaves are carefully teased away with needles. Free-hand sectioning with a sharp razor and judicious teasing with a pair of needles will give a fair insight into the anatomy of the higher plants without demanding any further knowledge of technique. This rough work is a very desirable antecedent to the study of microtome sections, because most students see in a series of microtome sections *only* a series of sections when, in the mind's eye, they ought to see the object building itself up in length, breadth, and thickness as they pass from one section to another.

CHAPTER IV.

THE GENERAL METHOD.

We shall now consider the routine of mounting an object in Canada balsam. While the outline refers more particularly to the paraffin method, the principles are general in their application and must be mastered by everyone who desires to make first-class preparations. Several of the topics, like killing and fixing, staining, etc., will be treated in detail when considering the various reagents.

I. KILLING AND FIXING.

Usually the same reagent is used for both killing and fixing. The purpose of a killing agent is to bring the life-processes to a sudden termination, while a fixing agent is used to fix the cells and their contents in as nearly the living condition as possible. The fixing consists in so hardening the material that the various elements may retain their natural condition during all the processes which are to follow. This step is one of extreme importance. Take the killing and fixing fluids into the field. If one waits until the material is brought to the laboratory, there may be some fixing, but it will, in many cases, be too late to do much killing. Material like *Spirogyra*, however, may be brought from the field into the laboratory before fixing, if considerable water be brought with it. Branches with developing buds may be brought in and kept in water. Always have the material in very small pieces, in order that the reagents may act quickly on all parts of the specimens. Pieces larger than one-fourth inch cubes should be avoided whenever possible. For very fine work no part of the specimen should require the reagent to penetrate more than one-sixteenth of an inch. In general, the volume of the reagent should be ten to fifty times that of the material. The time

required for this process varies with the reagent, the character of the tissue, and the size of the piece. About twenty-four hours is a commonly recommended period for chromic-acid solutions. While this might suffice in some cases, we should recommend two or three days, and even a longer period would probably do no harm.

II. WASHING.

Nearly all fixing agents, except the alcohols, must be washed out from the material as completely as possible before any further steps are taken, because some reagents leave annoying precipitates which must be removed, and others interfere with subsequent processes. Aqueous fixing agents with chromic acid as their principal ingredient are washed out with water; aqueous solutions of corrosive sublimate are also washed out with water; but alcoholic solutions should be washed out with alcohol of about the same strength as the fixing agent; picric acid, or fixing agents with picric acid as an ingredient, must not be washed out with water, but with alcohol, whether the picric acid be in aqueous or alcoholic solution. Running water is best, and where this is not convenient the water should at least be changed quite frequently. The washing-out process usually takes from twelve to twenty-four hours, but it can be shortened about one-half by keeping the fluid lukewarm.

III. HARDENING AND DEHYDRATING.

After the material has been washed, it is necessary to continue the hardening and also to remove the water. Alcohol is used almost entirely for these purposes. It completes the hardening and at the same time dehydrates, that is, it replaces the water in the material, an extremely important consideration, for the least trace of moisture, a trace so slight as to be almost imaginary, is nevertheless sufficient to make a preparation poor or indifferent when it might have been excellent.

The process of hardening and dehydrating must be gradual. If the material should be transferred directly from water to absolute alcohol, the hardening and dehydrating would be

brought about in a very short time, but the violent osmosis would cause a ruinous contraction of the more delicate parts. Therefore, transfer from water to 35 per cent. alcohol, which should act for six to twenty-four hours. Then use 50 per cent. for a similar period. Material may now be placed in 70 per cent. alcohol, where it may remain until ready for use, since 70 per cent. alcohol is a good preservative. Various devices, like constant drips and osmotic apparatus, have been proposed to secure a more gradual transfer. Whether these have any real advantages still remains to be proved. The writer has taken well-fixed fern prothallia through the series 35 per cent., 50 per cent., 70 per cent., without the slightest plasmolysis. Such things as fern prothallia, filamentous algæ, etc., can be watched under the microscope as the transfer is made, and, if plasmolysis results, the series of alcohols may be made closer, *e. g.*, 10 per cent., 20 per cent., 30 per cent., etc. It is said that material left for some time in 70 per cent. alcohol will shrink in spite of good killing and fixing, and it is also claimed that its capacity for staining is diminished. Some recommend that glycerine be added to the alcohol; others prefer to complete the dehydrating process and leave the material in an essential oil; while still others would imbed it and keep it in paraffin. The last is doubtless best of all, but requires such an immense amount of labor that it is impracticable for general purposes. Nearly all of our own material which is not needed for immediate use is in 70 per cent. alcohol, unless, of course, the material has been put into formalin or some such reagent which kills, fixes, and preserves all at once.

After the 70 per cent. alcohol, use 85 per cent. and 95 per cent., allowing six to twenty-four hours for each. Then use 100 per cent. alcohol for one or two days. The 70 per cent. would probably complete all the hardening which is necessary, but the other three must be used to complete the removal of water.

Up to this point the processes are exactly the same, whether the material is to be imbedded in paraffin or celloidin.

IV. CLEARING.

Let us suppose that the material has been thoroughly dehydrated, so that not the slightest trace of water remains. If the supposition chances to be contrary to fact, all the work which has preceded, as well as all which is to follow, is only an idle waste of time. The purpose of a clearing agent is to make the tissues transparent, but clearing agents also replace the alcohol. At this stage the latter process is the essential one, the clearing which accompanies it being incidental. The clearing, however, is very convenient, since it shows that the alcohol has been replaced and that the material is ready for the next step.

Various clearing agents are in use. Xylol is the most generally employed, and for most purposes it seems to be the best. Bergamot oil, cedar oil, clove oil, turpentine, and chloroform are all necessary for special purposes.

The transfer from absolute alcohol to the clearing agent should be *gradual*, like the hardening and dehydrating processes. The following is a good method:

3 parts 100 per cent. alcohol and 1 part xylol, 1 to 10 hours.
2 parts 100 per cent. alcohol and 2 parts xylol, 1 to 10 hours.
1 part 100 per cent. alcohol and 3 parts xylol, 1 to 10 hours.

This transfer is best accomplished by adding the xylol to the alcohol. It is not necessary to measure, since anyone can guess with sufficient accuracy. Shake gently each time the xylol is added. Pour off the mixture and add pure xylol, which should cause the material to become transparent. This may require only a moment, but may require hours. Other clearing agents may be used in the same way instead of the xylol.

V. THE TRANSFER FROM CLEARING AGENT TO PARAFFIN.

This should also be a *gradual* process. The most convenient method is to place a small block of paraffin in the pure clearing agent with the material. The paraffin dissolves gradually and produces the same result as if a small shaving of paraffin had been added every few minutes for a day or so. During this process the bottle or dish should be kept lukewarm. Six to ten hours, or over night, is usually sufficient for this step,

although it would seem that material may be kept here for a much longer time without injury, and in case of such refractory material as the megaspores of the heterosporous pteridophytes four or five days seem necessary. Excellent preparations of the embryo-sac of *Aster* have been made from material which had remained in the xylol and paraffin for nearly three years. No more paraffin should be added than will go into perfect solution. The temperature may be gradually increased, so that a much greater amount of paraffin will go into solution, but the paraffin must not be allowed to crystallize.

VI. THE PARAFFIN BATH.

This step is usually called infiltration, but when the transfer from the clearing fluid to paraffin is made gradually, as has just been indicated, the process of infiltration is already begun. It is now necessary to get rid of the xylol or other clearing agent. This may be done by simply pouring off the mixture of xylol and paraffin and replacing it with pure melted paraffin. The bath should be kept at a temperature about 1° C. higher than the melting-point of the paraffin. Fifty-three degrees C. is a good temperature for general purposes, but this may be reduced from 1° to 3° C. in winter, and must often be raised in summer. For special purposes it is sometimes necessary to use a temperature as high as 70° C. If the xylol or other clearing agent is not thoroughly removed, the paraffin will be granular or mealy, and will not cut well. The paraffin should be changed once or twice to make sure that the clearing agent is all removed. Do not waste this paraffin, for the clearing agent can be driven off by prolonged heating, and the paraffin will be better than ever. Most people use soft paraffin (about 45° C.) for the first half of the time necessary for infiltration, and a harder grade (48° C. to 54° C.) for the latter part of the process. This is a good plan, for soft paraffin melts at a lower temperature, and it is always well to minimize heat.

Some think that it is better not to pour off the mixture of the clearing agent and paraffin, but rather to evaporate the clearing

agent by keeping the temperature just high enough to prevent the crystallization of the paraffin. This method has certainly given good results with small, delicate objects.

The time required for infiltration varies with the character of the tissue and the size of the piece. Few things can be well infiltrated in less than an hour. Lily ovaries require one to four hours; heads of *Aster* at the fertilization period, six to twelve hours. Some claim that even delicate objects, like fern prothallia and the filamentous algæ and fungi, are not injured by a bath of several days, if care be taken not to let the temperature rise above 48° C. to 50° C. No one seems to know how long a certain object should remain in the bath, but many competent investigators are now using more prolonged periods.

VII. IMBEDDING.

Material may be imbedded in paper trays, watch crystals, or in apparatus made for the purpose. Imbedding L's consisting of two L-shaped pieces (*fig. 11*) of brass, type metal, or lead are very convenient. We use a pair-of L-shaped pieces with arms three inches long. These furnish a box of almost any required size. A piece of glass serves for a bottom. The tray, minot, or whatever is used, should be slightly smeared with glycerine, to prevent sticking. If several objects are to be imbedded in one dish, it is best to have the dish as near the temperature of melted paraffin as possible; otherwise the objects may stick to the bottom, and it will be impossible to arrange them properly. Great care should be taken, however, not to have the dish too hot, since too high a temperature not only injures the material, but also prevents a thorough imbedding. Pour the paraffin with the objects into the imbedding dish and cool as rapidly as possible. If paraffin cools slowly, it crystallizes and does not cut well. The layer of paraffin should be just thick enough to cover the objects, not only as a matter of economy, but because a thick layer retards the cooling. Very

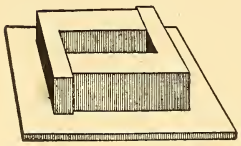

FIG. 11.

small objects, like the megaspores of *Marsilea*, ovules of *Silphium*, etc., may simply be poured out upon a *cool* piece of glass. In this way very thin cakes are made, which harden very rapidly.

VIII. CUTTING.

Twenty minutes after an object is imbedded it is ready for cutting. Trim the paraffin containing the object into a convenient shape, and fasten it upon a block of wood. Blocks of pine three-fourths of an inch long and three-eighths of an inch square are good for general purposes. Put paraffin on the end of the block so as to form a firm cap about one-eighth of an inch thick. Warm the cap and the bottom of the piece containing the object, and press them lightly together; then touch the joint with a hot needle, put the whole thing into cold water for a minute, and it is ready for cutting. Cutting can be learned only by experience, but a few hints may not come amiss: (*a*) Keep the knife *sharp*. If expense is not too serious an objection, it is well to have two hones, one rather soft, for use when the knife is dull, and the other quite hard, for putting on an even edge. Flood the stone with water, and rub it with the small slip which accompanies all high-grade hones; this not only makes a lather which facilitates the sharpening, but it also keeps the surface of the hone flat. As soon as the edge of the knife appears smooth and even under a magnification of thirty or forty diameters, the sharpening is completed with a good strop. It is better to sharpen the knife every time you use it. (*b*) Keep the microtome well oiled and *clean*. (*c*) Trim the block so that each section shall be a *perfect rectangle*.

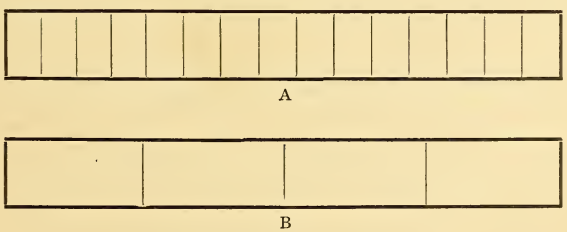

FIG. 12.

A ribbon of sections like that shown in *fig. 12 A* is much better than one like *B* of the same figure, because sections will usually come off in neater ribbons if the knife strikes the longer edge of the rectangle, so that the sections are united by their longer sides rather than by the shorter. Crooked ribbons are caused by wedge-shaped sections, and are always to be avoided, because they make it difficult to economize space, and also because they present such a disorderly appearance. The knife, which should be placed at a right angle to the block and not obliquely, should strike the *whole edge* of the block at once, and should leave in the same manner.

IX. FIXING SECTIONS TO THE SLIDE.

Sections must be firmly fixed to the slide, or they will be washed off during the processes involved in staining. Mayer's albumen fixative is excellent for this purpose. Formula:

White of egg, 50 cc. (Active principle.)
Glycerine, 50 cc. (To keep it from drying up.)
Salycilate of soda, 1 g. (Antiseptic, to keep out bacteria, etc.)

Shake well and filter. It will keep from two to six months. The fixative may be used alone or in connection with the water method. Put a small drop of fixative on the slide, smear it evenly over the surface, and then wipe it off with a clean finger or piece of linen until only a scarcely perceptible film remains; then add several drops of distilled water and float the sections or ribbons on the water. Warm gently until the paraffin becomes smooth and free from wrinkles. Be careful not to melt the paraffin, for the albumen of the fixative coagulates with less heat than is required to melt the paraffin. It is a very good plan to put the slide on the top of the water bath for a moment and then, after the sections have become smooth, remove the surplus water and leave them on the bath with a couple of thicknesses of blotting paper under them for three or four hours, or, better, over night. If the fixative is used alone, as is often the case when sections are very thick, none of this delay is necessary, since the sections are merely laid upon the fixative and pressed down gently with the finger.

X. REMOVAL OF THE PARAFFIN.

To remove the paraffin it is very customary to heat the slide gently until the paraffin melts, and then place the slide in xylol for thirty seconds or a minute. I believe, however, that it is far better merely to warm the slide a little (not warmer than 30° C.). Good xylol will then remove the paraffin in two or three minutes. Even if the slide should not be warmed at all, good xylol should remove the paraffin in five minutes.

XI. REMOVAL OF XYLOL.

The xylol may be removed either by absolute alcohol or by the 95 per cent. The absolute alcohol does not seem to be really necessary. For my own work I have two stender dishes of xylol and two of absolute alcohol. After the xylol has been used for a time I employ it only for removing paraffin, and in the same way use the absolute alcohol only for removing the xylol, while the other two dishes are used only for dehydrating and clearing. In transferring from xylol to absolute alcohol, or *vice versa*, it is well to drain off the superfluous liquid by resting the corner of the slide upon a piece of blotting paper. These rather expensive reagents will last much longer when this precaution is taken.

XII. TRANSFER TO THE STAIN.

Stains are aqueous or alcoholic, and alcoholic stains are of various strengths. If an aqueous stain be used, the slide should be passed successively through the alcohols 95 per cent., 85 per cent., 70 per cent., 50 per cent., and 35 per cent., allowing each to act for about thirty seconds, after which the slide is put into the stain. (In many cases it is sufficient to put a few drops of the stain on the slide with a pipette.) From the stain the slide is passed back through the various grades of alcohol, allowing it to remain about thirty seconds in each as before. If the stain be alcoholic of about 70 per cent. strength, the process is somewhat shorter, for the slide goes into the stain from 70 per cent. alcohol, and goes back into 70 per cent. alcohol from the stain. The rule is to transfer to the stain from the alcohol

which is nearest the strength of the stain. These directions are based upon a rather wide experience and certainly give excellent results with stains which do not forbid their application. However, evidence has long been accumulating which indicates that in case of very thin sections (sections so thin that no whole cells are included) only the absolute alcohol is necessary and that the rest may be omitted. Whether it is better to omit the alcohols from 35 per cent. to 95 per cent. when staining on the slide is a question still waiting for a definite answer, although careful and systematic experimenting would soon settle it. Even if it should be shown that it is as well or better to omit the alcohols from 35 per cent. to 95 per cent. in case of thin sections, it must not be inferred that these alcohols may be omitted in preparing a piece of epidermis stripped from a leaf, for, in this case, a ruinous plasmolysis occurs, and in sections thick enough to include whole cells the same damage is done. In the directions given in this book it is assumed that the series 35 per cent., 50 per cent., 70 per cent., 85 per cent., 95 per cent., and 100 per cent. is to be used, unless otherwise mentioned.

XIII. CLEARING.

After the sections have been stained, passed back through the various grades of alcohol, and have been thoroughly dehydrated in absolute alcohol, they are cleared or made transparent by means of xylol or some other clearing agent. The clearing agent must be a solvent of balsam. From thirty seconds to five minutes will be sufficient for clearing any kind of sections.

XIV. MOUNTING IN BALSAM.

After the sections are cleared, wipe the slide on the side which does not bear the sections. Put on a drop of Canada balsam and add a clean,[1] thin cover. Before the cover is put on, pass it through the flame of an alcohol lamp to remove moisture, for it would be a pity indeed to injure a preparation

[1] Slides and covers should be treated with hydrochloric acid, or equal parts of hydrochloric acid and water, for several hours. They should then be thoroughly rinsed in water and placed in 95 per cent. alcohol. They should be wiped with a cloth perfectly free from lint.

at this stage of the process. Add a label, and the mount is complete.

A TENTATIVE SCHEDULE FOR PARAFFIN SECTIONS.

It will be useful to give several tentative schedules for the use of beginners. It cannot be too strenuously insisted that *these schedules are only tentative*, their sole object being to give the beginner a start. The following is a tentative schedule for the ovary of a lily at any period before fertilization. The pieces should not be more than half an inch in length.

1. Chromo-acetic acid, 2 days.
2. Wash in water, 1 day.
3. Thirty-five per cent., 50 per cent., 70 per cent., 85 per cent., 95 per cent. alcohol, 6 to 24 hours each, as convenient.
4. One hundred per cent. alcohol, 24 hours. This should be changed once or twice. The volume should be at least ten times that of the material.
5. Transfer from absolute alcohol to xylol, allowing at least 2 hours in each of the three mixtures, and 2 hours in pure xylol.
6. Add paraffin to the xylol and keep warm for 12 to 24 hours.
7. Melted paraffin in the bath, 2 to 24 hours, as convenient. The paraffin should be changed once or twice.
8. Imbed.
9. Section; about 10 μ is a good thickness.
10. Fasten to the slide.
11. Dissolve off the paraffin in xylol.
12. One hundred per cent., 95 per cent., 85 per cent., 70 per cent., 50 per cent. alcohol, 30 seconds each. An hour in each would do no damage if other duties should interfere.
13. Delafield's hæmatoxylin, 10 minutes.
14. Rinse in water, 5 minutes. A couple of hours does no harm.
15. Thirty-five per cent., 50 per cent., and 70 per cent. alcohol, 30 seconds each. An hour would do no harm.
16. Acid alcohol, 1 second.
17. Seventy per cent. alcohol, 1 minute. An hour would do no harm.
18. Erythrosin, 30 seconds to 1 minute.
19. Eighty-five per cent., 95 per cent., 100 per cent., about 5 seconds each. This step must not be too prolonged, lest the erythrosin wash out.
20. Xylol, at least 1 minute. The slide may be left here for an hour without injury.
21. Mount in balsam.

On the whole, it is not a good plan to use protracted periods in the processes from No. 11 to No. 20 inclusive, because there is danger that a prolonged soaking may loosen some of the more delicate contents of the cells, even if whole sections do not come off bodily.

CHAPTER V.

KILLING AND FIXING AGENTS.

In this short account only the reagents which are at present considered most valuable for botanical work will be considered. Probably no process in microtechnique is in more urgent need of improvement than this first step of killing and fixing. Nearly all of our formulæ are merely empirical, for very few botanists are expert chemists, and those who have the requisite knowledge of chemistry are interested in physiological problems rather than in microtechnique. The principal ingredients of the usual killing and fixing agents are: alcohol, chloroform, chromic acid, acetic acid, osmic acid, formic acid, picric acid, sulphuric acid, platinum chloride, iridium chloride, corrosive sublimate, and formalin. We shall consider first

THE ALCOHOLS.

a. **Ninety-five per cent. Alcohol.**—This is in quite general use for material which is needed only for rough work. It is extremely convenient, since it kills, fixes, and preserves at the same time and needs no changing or washing. It really has nothing to recommend it for fine work. It causes protoplasm to shrink, but cell walls usually retain their position, so that 95 per cent. alcohol will do for free-hand sections of wood and many herbaceous stems; but even free-hand sections of tender stems, like young geraniums and begonias, will look better if better reagents are employed. Alcohols weaker than 95 per cent. are not to be recommended as fixing agents, although 70 per cent. alcohol, or even 50 per cent., will preserve material for habit work.

b. **Absolute (100 per cent.) Alcohol.**—This is a fair killing and fixing agent, but is rather expensive. It causes but little shrinking of the protoplasm, and is a time-saver if material is to be imbedded in paraffin. With 95 per cent. or with absolute

alcohol, objects are generally left in the reagent until needed for use, but such material becomes very brittle. The addition of glycerine is an improvement, if material is to be kept long. Acetic acid has been used with alcohols to counteract the tendency to shrink. One of the most successful of the alcohol combinations is

c. **Carnoy's Fluid.**—

Absolute alcohol, 6 parts.
Chloroform, 3 parts.
Glacial acetic acid, 1 part.

The penetration of the reagent is excellent, and only a few hours are needed for fixing. Material should be washed in absolute alcohol (perhaps 95 per cent. alcohol would do no harm) until there is no odor of acetic acid. This should not require more than one or two hours. It is better to imbed in paraffin at once, but when this is not convenient the material may be transferred to 85 per cent. alcohol and then to 70 per cent., where it may be left until needed. Cyanin and erythrosin, fuchsin and iodine green, and similar combinations, give particularly brilliant staining after this reagent.

THE CHROMIC-ACID GROUP.

Chromic acid, or solutions with chromic acid as a foundation, are the most generally useful killing and fixing agents yet known to the botanist. A 1 per cent. solution of chromic acid in water gives good results, but it is better to use the chromic in connection with other ingredients, such as acetic acid, formic acid, osmic acid, etc. The proportions of the various ingredients, for the present at least, must be determined by experiment. With favorable objects like fern prothallia, *Spirogyra*, and other things which can be watched while the fixing is taking place, suitable proportions are rather easily determined, because specimens, after being placed in the reagent, may be examined at frequent intervals, and combinations which cause plasmolysis may be rejected and different proportions tried until satisfactory results are secured. For example, fern prothallia might be placed in the following solution: chromic acid, 2 g.; acetic acid, 1 cc.;

and water, 97 cc. If plasmolysis takes place, weaken the chromic or strengthen the acetic, since the chromic has a tendency to produce contraction, and the acetic to cause swelling. Too large a proportion of acetic acid, however, may cause distortion, and hence it would be better to weaken the chromic. In case of fern prothallia, 3 parts chromic, 1 part acetic, and 396 parts water will cause practically no plasmolysis, and the fixing is sufficiently thorough to permit imbedding in paraffin. A combination may be quite satisfactory for fern prothallia and still fail to give good results with *Spirogyra*, and a combination which succeeds very well with *Spirogyra* may not succeed at all with *Vaucheria*. For very critical work the most favorable proportions must be determined for the particular plant under investigation. When the effect of the reagent cannot be observed directly, it is well to make a free-hand section and thus determine whether plasmolysis takes place. It is not safe to judge the action of a fixing agent by the appearance of sections cut from material which has been imbedded in paraffin, because shrinking of the cell contents often takes place during the transfer from absolute alcohol to the clearing agent or during infiltration with paraffin, and sometimes during even later processes. When in doubt as to proportions, we should suggest 2 cc. chromic acid, 2 cc. acetic acid, and 296 cc. water as a good formula for most purposes.

The time required for fixing undoubtedly varies with different plants, but twenty-four hours may be considered a minimum even for the most delicate objects. It is well known that zoölogists allow fixing agents like Müller's fluid and Erlicki's fluid to act for weeks before the material is passed on to the next stage, and it may well be questioned whether botanists have not made a mistake in allowing the chromic solutions to act for so short a time. At present most botanists recommend sixteen to twenty-four hours for material which is to be imbedded in paraffin, but some recent experiments in my laboratory indicate that material which has been in the fixing fluid for two or three days is better able to withstand the subsequent processes. More rapid penetration, and consequently more immediate killing, can

be secured if the reagent is kept warm (30° to 40° C.). Since chromic acid has a tendency to render objects hard and brittle, it is often better to use some other fixing agent, if much difficulty is anticipated in the cutting.

After fixing is complete, the reagent should be washed out with water. Running water is desirable, and where this is not convenient the water must be changed frequently. Any material should be sufficiently washed in six to twenty-four hours, but the time may be shortened about one-half by using lukewarm water.

Some of the formulæ are as follows:

a. **Strong Chromo-Acetic Solution.**—

1 g. chromic acid.
1 cc. glacial acetic acid.
98 cc. water.

This solution has been used quite extensively in embryological work upon the higher plants.

b. **Weak Chromo-Acetic Solution.**—(Schaffner's formula):

0.3 g. chromic acid.
0.7 acetic acid.
99 cc. water.

This has also been used in embryological work. It causes little or no plasmolysis, but the chromic seems rather weak. Difficult material, like *Aster* heads and ripe *Capsella* pods, cuts more readily after this reagent than after the stronger solution.

c. **Medium Chromo-Acetic Solution.**—

0.7 g. chromic acid.
0.5 cc. glacial acetic acid.
100 cc. water.

We are now using this solution, and it seems very successful in nearly all cases.

d. **Flemming's Fluid.**—(Weaker solution.)

A. { 1 per cent. chromic acid, 25 cc.
   { 1 per cent. acetic acid, 10 cc.
   { Water, 55 cc.
B. 1 per cent. osmic acid, 10 cc.

Keep the mixture A made up, and add B as the reagent is needed for use, since it does not keep well. This fluid is quite expensive on account of the osmic acid. For cytological work it gives as good results as any fixing agent which has yet been thoroughly tested. It is especially recommended for chromosomes, centrosomes, achromatic structures, and mitotic phenomena in general. Material should be in very small pieces one-eighth of an inch square, or in thin slices one-eighth of an inch or less in thickness, for the fluid penetrates poorly. The blackening due to the osmic acid may be removed by peroxide of hydrogen just before the slide is passed from the alcohol into the stain. Flemming's safranin-gentian violet-orange combination gives its most brilliant results after this reagent. It seems possible that the traditional superiority of this reagent has been overestimated.

e. **Merkel's Fluid.**—

Equal volumes of a 1.4 per cent. solution of chromic acid and a 1.4 per cent. solution of platinic chloride.

This is also an expensive reagent. It is recommended for mitotic phenomena, but does not seem to equal Flemming's solution.

f. **Hermann's Fluid.**—

1 per cent. platinic chloride, 15 parts.
Glacial acetic acid, 1 part.
2 per cent. osmic acid, 4 or 2 parts.

This is the most expensive fixing agent yet discovered, and for botanical purposes it does not seem to be any better than the cheaper chromic mixtures. It is mentioned here with chromic mixtures because it originated as a variation of Flemming's fluid, the platinic chloride being substituted for the chromic acid.

According to Lee, the chief objection to all mixtures into which chromic acid enters is that "it precipitates certain of the liquid albuminoids of the tissues in the form of filaments or network, which are often of great regularity and simulate structural elements of the tissues." Nevertheless, the mixtures which have

just been described are the best which have yet been thoroughly tested. If material killed in any of the above mixtures is not well washed, the hæmatoxylins will not stain. It is claimed that the anilins will stain in spite of poor washing, but it is a question whether such preparations are as permanent as those from well-washed material.

PICRIC ACID.

Use a saturated solution in water or 70 per cent. alcohol. One gram of picric acid crystals will saturate about 75 cc. of water or alcohol. This reagent penetrates well and does not make the material brittle. It is to be recommended when difficulty is anticipated in the cutting. If used cold, the time varies from one to twenty-four hours, depending upon the character of the tissue and size of the specimen. If used hot (85° C.), five or ten minutes will be sufficient. Material should be washed in 70 or 50 per cent. alcohol. Water is injurious, and some even go so far as to avoid aqueous stains, unless the material has been thoroughly washed. The washing should be continued until the material appears whitish and the alcohol no longer becomes tinged with yellow. Picro-carmine gives its best results after this reagent. Picric acid can be combined with various other fixing agents, and so we have picro-sulphuric acid, picro-nitric acid, picro-chromic acid, picro-chromic-sulphuric acid, and picro-osmic acid.

CORROSIVE SUBLIMATE.

Use a 2 to 5 per cent. solution in water, or 70 per cent. alcohol. The addition of about 1 cc. of glacial acetic acid to 100 cc. of this solution is certainly an improvement. The time required is considerably shorter than for chromic solutions. From one to ten hours will be found to be sufficient. If used hot (85° C.), only five or ten minutes is required. Washing may be done with water, but 50 per cent. alcohol is better. If a few drops of an iodine solution be added to the alcohol, the alcohol takes on a brownish color, but soon clears up. If the addition of iodine be continued, the washing is complete when the alcohol no longer clears up, but retains the brown color. If the washing is

incomplete, crystals of corrosive sublimate will be unduly conspicuous in preparations made from the material. Camphor may be used instead of iodine to hasten the washing.

The carmines are very brilliant after corrosive sublimate on account of the formation of mercuric carminate, but hæmatoxylin and anilines also give good results. It is claimed, however, that achromatic structures do not stain well with the safranin-gentian violet-orange combination after this fixing agent, but it might be worth while to test other stains before discarding this reagent for cytological work.

Corrosive sublimate material gets very brittle if allowed to remain long in alcohol, and therefore it is better to imbed it as soon as possible.

FORMALIN.

Formalin is a comparatively recent addition to the list of killing and fixing agents. It is an excellent preservative, often preserving the blue and red colors as well as the structure of objects. A 2 or 4 per cent. solution in water is good for filamentous algæ. The material may simply be put into the reagent and left until needed for use. For class use formalin material should be washed in water for several minutes, because the fumes are irritating to the eyes and mucous membranes. After a thorough washing in water any of the usual stains may be used.

GENERAL HINTS ON FIXING.

It is very desirable that the fixing agent should penetrate quickly to all parts of the object. For this reason material should be in small pieces. The best fixing agents do their best work near the surface of the piece. Small objects like *Azolla* and fern prothallia may be thrown into the fixing agent entire; even larger objects, which, like the anthers of *Lilium*, are easily penetrated, may also be put in without any cutting. Most objects larger than quarter of an inch cubes should be trimmed with a sharp knife or razor; some knowledge of the structures concerned is essential before one can trim material with unvarying success.

Some objects, although small, cause trouble in various ways. Many buds are hairy and will not sink; if such things are dipped quickly in strong alcohol, they will usually sink. If rather large air bubbles prevent the material from sinking, as in case of perichaetical leaves of some mosses and involucral leaves of liverworts, a little dissection or a careful snip with the scissors will obviate the difficulty. If an air-pump is available, the bubbles are easily removed.

It is often asked whether fixing agents really preserve the actual structure of cell contents. It must be admitted that some things, notably the liquid albuminoids, are much modified in appearance, but the most competent observers are now inclined to believe that such delicate objects as chromosomes, centrosomes, the achromatic figure, and even the structure of protoplasm, can be studied with confidence from material which has been fixed, imbedded, and stained. Recent study of these objects in the living condition has strengthened this confidence.

It is certain that we have not yet found the ideal fixing agent for cell contents. Such an agent must not be a solvent of any of the cell contents, must penetrate rapidly, must preserve structures perfectly, and must harden so thoroughly that every detail shall remain unchanged during the subsequent processes of dehydrating, clearing, imbedding, sectioning, and staining.

CHAPTER VI.

STAINING.

Staining is one of the most important and most complicated processes of microtechnique. The formulæ are largely empirical, and there is still abundant room for experimentation in the use of mordants and in the effect of the same stain or combination after various fixing agents.

Stains may be classified in various ways; *e. g.*, there are three great groups of stains—the Carmines, the Hæmatoxylins, and the Anilins. Stains may be classified as basic and acid, or they may be regarded as general and specific. A general stain affects all the elements, while a specific stain affects only certain elements or stains some elements more deeply than others. Stains which show a vigorous affinity for the nucleus have been called nuclear stains, and those which affect the cytoplasm more than the nucleus have been termed plasma stains. Of course, such stains are specific.

We shall consider some of the more important hæmatoxylins, carmines, and anilins, reserving general directions and theoretical questions until the end of this chapter. Many of the formulæ are taken from *The Microtomist's Vade-Mecum* (Lee), which is easily the most complete compendium of stains and other reagents concerned in microtechnique. It is to be regretted that botanists have no book of this character, but it must be confessed that we have not the material for such an extensive work. Other formulæ are from *Botanical Microtechnique* (Zimmermann) and from Stirling's *Histology*. The directions for using a stain apply to stains made up according to the formulæ which are given here, and may need modification if other formulæ are employed. It is hoped, however, that the directions will give the student sufficient insight into the *rationale* of staining to enable him to make any necessary modifications.

THE HÆMATOXYLINS.

The most important hæmatoxylins are Delafield's hæmatoxylin, Kleinenberg's hæmatoxylin, Erlich's hæmatoxylin, Boehmer's hæmatoxylin, Mayer's hæm-alum, and Haidenhain's iron alum-hæmatoxylin.

All the hæmatoxylins mentioned above contain alum, and, according to Mayer, who has written the most important work on hæmatoxylin stains ("Ueber das Färben mit Hämatoxylin," *Mitth. a. d. Zool. Station zu Neapel*, **10**: 170–186, 1891, and "Ueber Hämatoxylin, Carmin und verwandten Materien," *Zeit. f. wiss. Mikr.*, **16**: 196–220, 1899). "The active agent in them is a compound of hæmatin with alumina. This salt is precipitated in the tissues, chiefly in the nuclei, by organic and inorganic salts there present (*e. g.*, by the phosphates) and perhaps also by other organic bodies belonging to the tissues." These salts are fixed in the tissues by the killing and fixing agent, and when the stain is applied a chemical combination results. Hæmatoxylins stain well after any of the fixing agents described in the preceding paper, but they are most effective when used after members of the chromic-acid series.

Delafield's Hæmatoxylin.—"To 100 cc. of a saturated solution of ammonia alum add, drop by drop, a solution of 1 g. of hæmatoxylin dissolved in 6 cc. of absolute alcohol. Expose to air and light for one week. Filter. Add 25 cc. of glycerine and 25 cc. of methyl alcohol. Allow to stand until the color is sufficiently dark. Filter, and keep in a tightly stoppered bottle." (Stirling and Lee.) The addition of the glycerin and methyl alcohol will precipitate some of the ammonia alum in the form of small crystals. The last filtering should take place four or five hours after the addition of the glycerine and methyl alcohol.

The solution should stand for at least two months before it is ready for using. This "ripening" is brought about by the oxidation of hæmatoxylin into hæmatin, a reaction which may be secured in a few minutes by a judicious application of peroxide of hydrogen.

Transfer to the stain from 35 per cent. alcohol or from water. The length of time required is exceedingly variable. Sometimes sections will stain deeply in three minutes, but it is often necessary to stain for thirty minutes or even longer. This stain may be diluted with several times its own volume of water; when this is done, the time required is correspondingly long, but the staining is frequently more precise. The length of time required will be fairly uniform for all material taken from the same bottle. This fact indicates that the washing process, which follows killing and fixing, is an important factor; if the washing has been thorough, the material will stain readily; but if the washing has been insufficient, the material may stain slowly or not at all. The washing is particularly important when the fixing agent contains an acid. Transfer from the stain to water. Distilled water is neither necessary nor desirable. Some writers recommend washing for twenty-four hours, but this seems unnecessary; an hour is usually enough, and a few minutes is often sufficient. Precipitates are often formed when slides are transferred directly to alcohol from this stain; otherwise, it would be better to transfer from the stain to 35 per cent. alcohol. Pass through the alcohols to 70 per cent. alcohol and then give the slide a few dips (two seconds is often sufficient) in acid alcohol (0.5 cc. HCl. to 100 cc. of 70 per cent. alcohol). This extracts the stain more rapidly from other parts than from the nuclei, and hence gives a good nuclear stain. Some prefer to stain for a very short time and use no acid alcohol, but, as a rule, it seems best to overstain and then differentiate in this way, because sharper contrasts are obtained. Transfer from acid alcohol to 70 per cent. alcohol and leave here until a rich purple color replaces the red due to the acid. Since small quantities of the acid alcohol are carried over into the 70 per cent. alcohol, it is well to add a *drop* of ammonia now and then to neutralize the effect of the acid. Too much ammonia is to be avoided, for it gives a disagreeable bluish color with poor differentiation, probably on account of the precipitation of alumina. The slide may now be passed through the alcohols, cleared in xylol, and mounted in balsam; or, if a double stain be

preferred, treat for thirty seconds to one minute with eosin, erythrosin, or some other stain affording a good contrast; rinse in 70 per cent. alcohol and proceed as usual.

Delafield's hæmatoxylin is the most generally useful stain in the hæmatoxylin group. It brings out cellulose walls very sharply, and consequently is a good stain for embryos and the fundamental tissue system in general. With safranin it forms a good combination for the vascular system, the safranin giving the lignified elements a bright red color, while the hæmatoxylin stains the cellulose a rich purple. It is a good stain for chromatin, and the achromatic structures show up fairly well, but can be brought out much better by special methods. Archesporial cells and sporogenous tissue are very well defined if proper care be taken. Whenever you are in doubt as to the selection of a stain for general purposes, we should advise the use of Delafield's hæmatoxylin.

The following is a general schedule for staining paraffin sections on the slide in Delafield's hæmatoxylin:

1. Stain (from water or 35 per cent. alcohol) 5 minutes.
2. Rinse in water, 5 minutes.
3. Thirty-five per cent., 50 per cent., and 70 per cent. alcohol, 30 seconds each.
4. Acid alcohol, 1 second.
5. Seventy per cent. alcohol, 1 minute.
6. Eighty-five per cent., 95 per cent., and 100 per cent. alcohol, 30 seconds each.
7. Xylol, 1 minute.
8. Mount in balsam.

If, after rinsing in water, the stain is evidently too weak, put the slide or section back into the stain until it appears overstained. For thin sections (10 μ or less) thirty seconds in each of the alcohols will be unnecessarily long, although thirty minutes would do no damage. Give the slide a single dip into the acid alcohol, transfer it quickly to the 70 per cent. alcohol, and then examine it; if it still appears overstained, give it another dip in the acid alcohol, and repeat the process until the stain is what you want. After the hæmatoxylin is just right, apply a contrast

stain, if you wish to double-stain. It is a good plan to move the slide gently to and fro in the absolute alcohol. Before transferring to the xylol, wipe the alcohol from the back of the slide, or at least rest the corner of the slide upon blotting paper for two or three seconds, in order that you may not carry over so much alcohol into the xylol, and thus impair this rather expensive reagent. The slide may also be moved gently to and fro in the xylol. Add a drop of balsam and a cover. Since the xylol is very volatile, this last step must be taken quickly. If blackish spots appear, they are usually caused by the drying of sections before the balsam and cover are added; if there are whitish spots or an emulsion-like appearance, the clearing is not thorough; this may be caused by poor xylol (or other clearing agent); by absolute alcohol which is considerably weaker than its name implies (the absolute alcohol must test at least as high as 99 per cent., and ought to test as high as 99.5 per cent., if xylol is to be used for clearing); or the difficulty may be caused by passing too quickly through the absolute alcohol and xylol, or may even be caused by moisture on the cover-glass.

Kleinenberg's Hæmatoxylin.— This stain has had a wide use, but it is now largely replaced by better formulæ. It is mentioned here merely because it is on the shelves of so many laboratories. It is doubtful whether it is equal to Delafield's in any kind of botanical work. A good description is given in the *Quarterly Journal of the Microscopical Society*, **74**: 208 —, 1897.

Erlich's Hæmatoxylin.—

Distilled water, 50 cc.
Absolute alcohol, 50 cc.
Glycerine, 50 cc.
Glacial acetic acid, 5 cc.
Hæmatoxylin, 1 g.
Alum in excess.

Keep it in a dark place until the color becomes a deep red. If well stoppered, it will keep indefinitely. Transfer to the stain from 50 per cent. or 35 per cent. alcohol. Stain five to thirty minutes. Since there is no danger from precipitates and the

solution does not overstain, it is not necessary to treat with water or with acid alcohol, but the slide may be transferred from the stain to 70 per cent. alcohol. Eosin, erythrosin, or orange G are good contrast stains.

Boehmer's Hæmatoxylin.—

A { Hæmatoxylin, 1 g.
  { Absolute alcohol, 12 cc.

B { Alum, 1 g.
  { Distilled water, 240 cc.

The solution A must ripen for two months. When wanted for use, add about 10 drops of A to 10 cc. of B. Stain ten to twenty minutes. Wash in water and proceed as usual.

Mayer's Hæm-Alum.—Hæmatoxylin, 1 g., dissolved with heat in 50 cc. of 95 per cent. alcohol and added to a solution of 50 g. of alum in a liter of distilled water. Allow the mixture to cool and settle; filter; add a crystal of thymol to preserve from mold. (Lee.)

It is ready for use as soon as made up. Unless attacked by mold, it keeps indefinitely. Transfer to the stain from water. It is seldom necessary to stain for more than ten minutes, and four or five minutes is generally long enough. As a rule, better results are secured by diluting the stain (about 1 cc. to 10 cc. of distilled water) and allowing it to act for ten hours or over night. This is a good stain for the nuclei of filamentous algæ and fungi, since it has little or no effect upon cell walls or plastids. Wash thoroughly in water and transfer to 10 per cent. glycerine. Specimens may be mounted in balsam, if they can be gotten through without shrinking.

Haidenhain's Iron Alum-Hæmatoxylin.—This stain was introduced by Haidenhain in 1892 and has gained a well-deserved popularity with those engaged in cytological work. Two solutions are used, and they are never mixed:

A. One and a half to 4 per cent. aqueous solution of ammonia sulphate of iron. (At present we use a 3 per cent. solution.)

B. One-half per cent. aqueous solution of hæmatoxylin.

The first solution acts as a mordant, *i. e.*, it does not stain, but prepares the tissue for the action of the second solution.

Transfer to the iron-alum from water; allow this solution to act for two hours; wash in water five minutes, and then stain in the ½ per cent. hæmatoxylin ten hours or over night. Rinse in water five minutes and treat for a second time with the iron alum, which now rapidly extracts the stain. The action of the iron alum should be watched under a microscope, and when the chromosomes of karyokinetic figures appear sharply defined, the slide should at once be placed in water and washed for at least an hour, since a very little of the iron alum, if left in the tissue, will cause the preparation to fade. If staining for details other than nuclei, the slide must be transferred to water as soon as the desired effect is produced. After the washing in water, the slide is passed through the alcohols, cleared in xylol, and mounted in balsam. This stain is excellent for the filamentous algæ and fungi, and it keeps well in glycerine. For preparations to be mounted in balsam, erythrosin, fuchsin, or orange G make good contrast stains. Apply the second stain after the last washing in water. The second stain should always be very light.

Chromosomes and centrosomes ("of those plants which have centrosomes") take a brilliant black, and other details, though not brightly colored, often show excellent definition.

The times given above must not be accepted as final. Many prefer to wash in water for several hours after the first immersion in iron alum. A plan which has proved convenient and very successful is to put the slide into the iron alum in the morning, let it wash in water during the afternoon, leave it in the ½ per cent. of hæmatoxylin over night, and finish the preparation the next morning. It is a long process, requiring care, patience, and judgment, but it is worth the effort.

THE CARMINES.

This group of stains, immensely popular a few years ago, has rapidly lost favor among botanists as newer stains and combinations have appeared. When it is desirable to stain in bulk, nothing has been found which will serve better than the carmines. Only three of these stains will be considered:

Greenacher's Borax Carmine.—

Carmine, 3 g.
Borax, 4 g.
Distilled water, 100 cc.

Dissolve the borax in water and add the carmine, which is quickly dissolved with the aid of gentle heat. Add 100 cc. of 70 per cent. alcohol and filter. (Stirling.)

The following is a slightly different method for making this stain from the ingredients mentioned above: Dissolve the borax in water, add the carmine, and heat gently for ten minutes; after the solution cools, add the alcohol and filter; let the solution stand for two or three weeks, then decant, and filter again.

Stain the material in bulk from 50 per cent. alcohol one to three days, then treat with acid alcohol (50 cc. of 70 per cent. alcohol + 2 drops of hydrochloric acid) until the color becomes a clear red; this may require only a few hours, but may take two or three days. The material may then be passed through the rest of the alcohols (six to twenty-four hours each), cleared, imbedded, and cut. After the sections are thoroughly fastened to the slide, the paraffin should be dissolved off with xylol. The balsam and cover may be added immediately, or the slide may be passed down through the alcohols for a contrast stain.

Alum Carmine.—A 4 per cent. aqueous solution of ammonia alum is boiled twenty minutes with 1 per cent. of powdered carmine. Filter after it cools. (Lee.)

Stain from water twelve to twenty-four hours and wash in water. No acid alcohol is needed, since the solution does not overstain.

Alum Cochineal.—

Powdered cochineal, 50 g.
Alum, 5 g.
Distilled water, 500 cc.

Dissolve the alum in water, add the cochineal, and boil; evaporate down to two-thirds of the original volume, and filter. Add a few drops of carbolic acid to prevent mold. (Stirling.)

Stain as with alum carmine. A few years ago it was a very common practice to stain in bulk in alum cochineal and counterstain on the slide with bismark brown.

THE ANILINS.

Many of the most brilliant and beautiful stains yet discovered belong to this group. These stains are so numerous that we shall not attempt to mention even their names, but shall consider only those which are in most common use by botanists. The following formula has proved to be fairly satisfactory for all the anilins mentioned in this account, but other formulæ will be given for most of the stains:

Make a 3 per cent. solution of anilin oil in distilled water; shake well and frequently for a day; add enough alcohol to make the whole mixture about 20 per cent. alcohol; add 1 g. of cyanin, erythrosin, safranin, gentian violet, etc., to each 100 cc. of this solution.

The anilins keep well in balsam, but not in glycerine. Xylol is a good clearing agent for all of them, but clove oil is very much better in case of gentian violet. Unfortunately, some of them do not give permanent stains. Some are acid, some basic, and some neutral.

The rapidity with which sections must be transferred from one fluid to another makes many of them more difficult to manage than the hæmatoxylins or the carmines, but the stains are so valuable that even the beginner should spend most of his time with the anilins.

Many anilins stain quite deeply in one to twenty minutes, but if the stain washes out during the dehydrating process, stain longer, even ten to thirty hours, if necessary. If the stains are made up according to the formula mentioned above, transfer to the stain from 35 per cent. alcohol and from the stain to 35 per cent. alcohol, if the stain does not wash out too rapidly; if the stain washes out, try 50 per cent., 70 per cent., 85 per cent., 95 per cent., or even directly to absolute alcohol. It will often be found impracticable to transfer from the stain to alcohols weaker

than the 85 per cent., lest all the stain be washed out before the clearing agent is reached.

Since the anilins are seldom used as single stains, but almost invariably in combination with other stains, the logical order will be disregarded and the stains will be treated, as they are used, in their most usual combinations.

Cyanin and Erythrosin.— Stain in cyanin five to ten minutes or longer; rinse quickly in alcohol, and then stain thirty seconds to one minute in erythrosin. If the cyanin washes out, stain for an hour, and if it still washes out, omit the rinsing in alcohol and transfer directly from the cyanin to the erythrosin.

The erythrosin may be used first; in this case stain for five minutes in erythrosin, transfer directly to cyanin, and stain for about ten seconds. Try 70 per cent. alcohol, but if the stains are lost, try 85 per cent., 95 per cent., or even transfer directly to 100 per cent.

This is a good combination for general work, and if properly used is excellent for mitotic phenomena and the most delicate cytological work. Delafield's hæmatoxylin may be used with erythrosin. Stain first with the hæmatoxylin, and, after the purple color has replaced the red due to the acid, stain lightly with erythrosin. Eosin may be used instead of erythrosin, but is less transparent. Eosin will be mentioned later in connection with special methods for algæ and fungi.

Flemming's Safranin-Gentian Violet-Orange. — Safranin has long been a famous stain for karyokinesis. This triple combination was published in 1891, but its value in plant cytology was not thoroughly appreciated until five or six years later, when its application was developed to a high degree of perfection by various investigators of the Bonn (Germany) school.

According to Flemming, stain two to three days in safranin (dissolve 0.5 g. safranin in 50 cc. absolute alcohol, and after four days add 10 cc. distilled water); rinse quickly in water; stain one to three hours in a 2 per cent. aqueous solution of gentian violet; wash quickly in water, and then stain one to three minutes in a 1 per cent. aqueous solution of orange G.

Tranfer from the stain to absolute alcohol, clear in clove oil, and mount in balsam.

The following method seems to be better for mitotic phenomena in plants: Transfer to safranin from 35 per cent. alcohol and stain sixteen to twenty-four hours. If the stain acts for only a few hours, it washes out too rapidly to be controlled with any precision. (The safranin may be made up according to the formula given in the preceding paragraph or according to the general formula.) Rinse in water for a minute and then in 50 per cent. alcohol until only nucleoli and the chromosomes of dividing nuclei retain the red color. If the alcohol does not wash out the stain sufficiently, dip the slide in acid alcohol (not more than 2 or 3 drops of hydrochloric acid to 100 cc. of 50 per cent. alcohol). If acid has been used, wash for a moment in pure 50 per cent. alcohol, and then stain for four or five minutes in gentian violet (aqueous solution, or made up according to the general formula). Rinse for a few seconds in water and then stain about thirty seconds in a 1 per cent. aqueous solution of orange G. Transfer from the stain directly to absolute alcohol and hasten the dehydrating by gently rinsing the slide in the fluid. One or two quick dips in the 95 per cent. alcohol before transferring to the absolute does not seem to do any harm and certainly saves the more expensive reagent. As a rule, not more than ten seconds can be allowed for dehydrating, because the gentian violet washes out so rapidly. The extreme transfer from the orange G, an aqueous stain, to absolute alcohol, which must often be made to prevent the gentian violet from washing out, indicates that, for staining very thin sections on the slide, the series of alcohols from 35 per cent. up to 100 per cent. is not at all necessary.

Treat with clove oil for five to ten seconds. The clove oil not only clears, but it rapidly extracts the gentian violet, producing an elegant differentiation. Replace the clove oil by cedar oil, if you have strictly first-class cedar oil. Such cedar oil is very thin, light yellow in color, and has a faint odor of cedar wood. It costs about the same as clove oil. Do not get

the expensive oil used for immersion lenses. If your cedar oil is colorless and has a strong odor, do not use it at all, but drain off the clove oil as thoroughly as possible by resting the edge of the slide on blotting paper for two or three minutes, and then mount in balsam. If any considerable amount of clove oil is left on the slide, the preparation is almost sure to fade. The cedar oil is not a solvent of gentian violet, and consequently there is little danger that the preparation will fade when this oil is used. Some investigators transfer from clove oil to xylol, but it seems to us that the brilliancy of the gentian violet is impaired. Chromosomes should take a clear red and the spindle fibers a bright violet. This combination is generally used after Flemming's solution, but seems to do equally well after other members of the chromic-acid series. Achromatic structures do not seem to stain well after corrosive sublimate or picric acid.

Fuchsin.—Use a 1 or 2 per cent. solution in water or in 70 per cent. alcohol. Transfer to the alcoholic solution from 70 per cent. alcohol; stain one to two hours; differentiate the stain in 1 per cent. solution of picric acid in 70 per cent. alcohol—this may require thirty seconds or several minutes; rinse in 70 per cent. alcohol until a bright red replaces the yellowish color due to the acid, and then proceed as usual.

Iodine Green.—A 1 per cent. solution in 70 per cent. alcohol is good for the vascular system of plants. This stain resists the washing-out process better than methyl green. Stain in iodine green at least an hour, and it is not a bad plan to stain over night; rinse in 70 per cent. alcohol; stain fifteen seconds to one minute in erythrosin, and proceed as usual. Methyl green may be made and used in the same way.

Fuchsin and Iodine Green Mixtures.—Two solutions are kept separate, since they do not retain their efficiency long after they are mixed:

A { 0.1 g. fuchsin (acid).
  { 50 cc. distilled water.

B { 0.1 g. iodine green.
  { 50 cc. distilled water.

C { 100 cc. absolute alcohol.
1 cc. glacial acetic acid.
0.1 g. iodine.

Mix equal parts of A and B. Transfer to the stain from water. The proper time must be determined by experiment. Twenty-four hours might be recommended for a trial. Transfer from the stain directly to solution C and from C to xylol.

Another formula:

A. 0.5 g. acid fuchsin.
B. 0.5 g. iodine green.

Mix a pipette full of A with a pipette full of B; stain two to eight minutes; transfer to 85 per cent. or 95 per cent. alcohol, dehydrate rapidly, clear in xylol, and mount in balsam. Both these formulæ are good for karyokinesis.

Bismark Brown.—Use a 2 per cent. solution in 70 per cent. alcohol. If material has been stained in bulk in one of the carmines, a few minutes' staining on the slide with bismark brown gives a good contrast. It is particularly good for cell walls.

Nigrosin.—Use a 1 or 2 per cent. solution in water. A few drops of this solution to a watch-glass full of water stains filamentous algæ or fungi in one to three hours. The stain keeps well in glycerine or balsam, but it is hard to get these forms into balsam without more or less shrinking.

CHAPTER VII.

GENERAL REMARKS ON STAINING.

It must be remembered that many things may be examined alive without killing, fixing, staining, or any of those processes. A filament of *Spirogyra* shows the chromatophore nicely if merely mounted in a drop of water; the nucleus may be visible, and the pyrenoids can usually be located. Of course, such a study is necessary if one is to understand anything about the plant, and in an elementary class this might be sufficient, but a drop of iodine solution applied to the edge of the cover would emphasize certain details, *e. g.*, the starch in the pyrenoids would appear blue, the nucleus a light brown, and the cytoplasm a lighter brown. This illustrates at least one advantage to be gained by staining; it enables us to see structures which would otherwise be invisible, or almost invisible.

With so many stains at our disposal, it at once becomes a problem just which stain or combination to use in each particular case. Beautiful and instructive preparations occasionally result from some happy chance, but uniform success demands skill and judgment in manipulation, and also a knowledge of the structures which are to be differentiated. Let us take a vascular bundle for illustration. Safranin stains the xylem a bright red, but, with judicious washing, is entirely removed from the cambium and cellulose elements of the phloëm. A careful staining with Delafield's hæmatoxylin now gives a rich purple color to the cellulose elements which were left unstained by the safranin, thus contrasting sharply with the lignified elements. If cyanin and erythrosin be used, the xylem takes the blue and the cambium and phloëm take the red. Many terms have been given to indicate the affinity of certain tissues for certain stains. Auerbach used the terms "erythrophilous" and "cyanophilous" in 1890. This eminent zoölogist studied spermatozoa and ova.

He found that, if preparations containing both spermatozoa and ova were stained with cyanin and erythrosin, the nuclei of the spermatozoa took the cyanin, while the nuclei of the ova preferred the erythrosin; hence the terms "cyanophilous" and "erythrophilous." Auerbach regarded these differences as an indication of sexual differences in the cells.

Rosen (1892) supported this theory, and even went so far as to regard the tube nucleus of the pollen grain as female, on account of its erythrophilous staining. In connection with this theory it was suggested that the ordinary vegetative nuclei are hermaphrodite, and that in the formation of a female germ nucleus the male elements are extruded, leaving only the erythrophilous female elements; and, similarly, in the formation of a male nucleus the female elements are extruded, leaving only the cyanophilous male elements.

As long ago as 1884 Strasburger discovered that with a mixture of fuchsin and iodine green the generative nucleus of a pollen grain stains green, while the tube nucleus stains red. In 1892 (*Verhalten des Pollens*) he discussed quite thoroughly the staining reactions of the nuclei. The nuclei of the small prothallial cells of gymnosperm microspores are cyanophilous like the male generative nuclei. The nuclei of a nucellus surrounding an embryo-sac are also cyanophilous, while the nuclei of structures within the sac are erythrophilous. His conclusion is that the cyanophilous condition in both cases is due to poor nutrition, while the erythrophilous condition is due to abundant nutrition. A further fact in support of the theory is that the nuclei of the adventitious embryos which come from the nucellus of *Funkia ovata* are decidedly erythrophilous, while the nuclei of the nucellus to which they owe their food-supply are cyanophilous.

In division stages nuclei are cyanophilous, but from anaphase to resting stage cytoplasm is taken into the nucleus, and the cyanophilous condition gradually changes to the erythrophilous.

An additional fact in favor of this theory is that in *Ephedra* the tube nucleus which has very little cytoplasm about it is

cyanophilous. Strasburger claims that there is no essential difference between male and female generative nuclei, and subsequent observation has shown that within the oöspore the sex nuclei are alike in their reaction to stains.

Malfatti (1891) and Lilienfeld (1892–3) claim that these reactions are dependent upon the amount of nucleic acid present in the structures. During mitosis the chromosomes consist of nearly pure nucleic acid and are intensely cyanophilous, but the protoplasm, which has little or no nucleic acid, is erythrophilous. There is a gradual transition from the cyanophilous condition to the erythrophilous, and *vice versa*, the acid structures taking basic stains and basic structures the acid stains.

The terms "erythrophilous" and "cyanophilous" are falling into disuse, since the affinity is for basic and acid dyes, rather than for blue or red colors. That the terms are misnomers becomes evident when a combination like safranin (basic) and acid green (acid) is used, for the cyanophilous structures stain red, and the erythrophilous green.

Probably but few investigators who have attained any proficiency in microtechnique have not asked themselves how much dependence can be placed upon staining reactions as a means of analysis. Do staining reactions enable us to determine the chemical composition of a structure? If two structures stain alike with Delafield's hæmatoxylin, does this mean that they have the same chemical composition; or if, on the other hand, they stain differently, must they necessarily be different in their chemical composition? Delafield's hæmatoxylin, when carefully used, gives a rich purple color, but a careful examination will often show that in the same preparation some structures stain purple, while others stain red. Does this mean that the purple and red structures must have a different chemical composition? Many people believe that structures which stain differently with a given stain must be chemically different, but they readily agree that structures which stain alike are not necessarily similar in chemical composition. Chromosomes of dividing nuclei and lignified cell walls stain alike with safranin;

chromosomes and cellulose cell walls stain much alike with Delafield's hæmatoxylin; but everyone recognizes that the chromosome is very different in its chemical composition from either the cellulose or the lignified wall.

According to Fischer (1897 and 1900), stains indicate physical but not chemical composition. Fischer experimented with substances of known chemical composition. Egg albumin was shaken until small granules were secured. These were fixed with the usual fixing agents, and then stained with Delafield's hæmatoxylin. The extremely small granules stained red, while the larger ones became purple. Since the granules are all alike in chemical composition, Fischer concluded that the difference in staining must be due to physical differences. With safranin, followed by gentian violet, the larger granules stain red and the smaller violet; if, however, the gentian violet be used first, then treated with acid alcohol and followed by safranin, the larger granules take the red and the smaller the gentian violet. In root tips similar results were obtained. Safranin followed by gentian violet stained chromosomes red and spindle fibers violet, while gentian violet followed by safranin stained the chromosomes violet and the spindle red. One often reads that chromosomes owe their strong staining capacity to nuclein, and especially to the phosphorus, but, according to Fischer, this is shown to be unfounded, since albumin gives similar results and yet contains no phosphorus, and is not chemically allied to nuclein. Delafield's hæmatoxylin is one of the so-called nuclear stains. The nuclei of animals and plants stain deeply with this reagent, but cellulose membranes, the dense protoplasm of embryonic cells, the pyrenoids of green algæ, and many other structures resemble nuclei in their staining. The most critical work on this subject has been done by those who are investigating the structure of the Cyanophyceæ and Bacteria to determine whether these forms have nuclei or not. Bütschli claims that the granules which stain red with hæmatoxylin are to be identified with chromatin, while Fischer, whose results have just been given, claims that staining indicates merely physical differences.

The subject cannot yet be regarded as settled, but whatever may be true in regard to these conflicting theories, all agree that stains are of the highest importance in differentiating structures, and in bringing out details which would otherwise be invisible.

CHAPTER VIII.

PRACTICAL HINTS ON STAINING.

In later chapters specific directions will be given for making a series of preparations ranging from the lowest algæ to the flowering plants, but a few suggestions will be made here.

The number of stains in the catalogues is becoming so great that it is impossible to become proficient in the use of all of them. It is far better to master a few of the most valuable stains than to do indifferent work with many. The beginner, especially if rather unacquainted with the details of plant structure, may believe that he has an excellent preparation when it is really a bad, or at most an indifferent, one. To illustrate, let us suppose that a pollen mother-cell in a late spirem stage has been stained with cyanin and erythrosin. A preparation in which the cell merely shows a differentiation into nucleus and cytoplasm must be classed as bad; if the nucleus shows a definitely outlined spirem thread, the preparation is better, but is still only indifferent; if the thread appears as a delicate red ribbon bordered by blue granules, the staining may be regarded as a success. If mitotic figures have been stained with cyanin and erythrosin, a first-class preparation should show blue chromosomes and red spindles; if stained with safranin and gentian violet, the chromosomes should be red and the spindles violet.

In staining growing points, apical cells, young embryos, antheridia, archegonia, and many such things, the cell walls are the principal things to be differentiated, if the preparations are for morphological study. As a rule, it is better in such cases not to use double staining, but to select a stain which stains the cell walls deeply without obscuring them by staining starch, chlorophyll, and other cell contents. For example, try the growing point of *Equisetum* The protoplasm of such growing points is very dense. If Delafield's hæmatoxylin and erythrosin be used,

the hæmatoxylin will stain the walls and nuclei, and will slightly affect the other cell contents, but the erythrosin will give the cytoplasm such a dense stain that the cell walls will be seriously obscured. It would be better to use hæmatoxylin alone. The same suggestion may well be observed in tracing the development of antheridia, archegonia, embryos, and similar structures.

Permanent preparations are an absolute necessity for the greater part of most advanced work, but let us not imagine that we cannot examine anything until we have made a permanent mount. It would be impossible to make a permanent mount of the rotation of protoplasm. It is better for many purposes to look at motile spores while they are moving. Use *Spirogyra* while it is fresh and green (if you can), and use permanent preparations only to bring out nuclei and other details which are not so easily seen in living material. Examples might be multiplied.

CHAPTER IX.

THE CELLOIDIN METHOD.

"Celloidin is a form of nitro-cellulose." It is very inflammable, but does not explode. It may be obtained in the form of tablets or cuttings, which have to be dissolved in a mixture of equal parts of absolute alcohol and ether. It is customary to use two solutions, a "thick" and a "thin." The thick solution (about 10 or 12 per cent.) should have about the consistency of thick syrup. The thin may be made by mixing equal parts of the thick and ether alcohol.

As mentioned in the chapter on the "General Method," the killing, washing, and dehydrating are the same as for the paraffin method. After dehydrating in absolute alcohol the succeeding steps are as follows:

1. Ether alcohol, 1 to 2 days.
2. Thin celloidin, 2 to 6 days.
3. Thick celloidin, 3 to 10 days.

It seems better, however, to begin with about 2 per cent. celloidin and transfer successively through 4 per cent., 6 per cent., etc., to 12 per cent., or to allow the 2 per cent. to concentrate by removing the cork for a short time each day.

The material may now be imbedded and mounted upon a block at the same time. The blocks should have surface enough to accommodate the objects, and should be about one-fourth of an inch thick. White pine makes good blocks; cork is much inferior. Place the block for a moment in ether alcohol and then dip into the 2 per cent. celloidin the end of the block which was left rough by the saw. With the forceps remove a piece of the material from the thick celloidin and place it upon the block, taking care to keep it right side up. Dip the block with its object first in thick celloidin, then in thin, and after exposing to the air for a few minutes drop it into chloroform,

where it should remain for about ten to twenty hours. It should then be placed in equal parts of glycerine and 95 per cent. alcohol, where it may be kept indefinitely. If the material is hard, like many woody stems, it will cut better after remaining in this mixture for a couple of weeks.

In cutting, the knife should be set as obliquely as possible, and both the knife and the object should be kept wet with the mixture of glycerine and alcohol. As fast as they are cut the sections are transferred with a soft brush to 70 per cent. alcohol. The succeeding steps are the same as for free-hand sections, but many stains are not available because they stain the celloidin. Safranin and Delafield's hæmatoxylin, or Delafield's hæmatoxylin and eosin, are good combinations for celloidin sections. Do not use absolute alcohol for dehydrating, since it dissolves the celloidin, but transfer from 95 per cent. alcohol to Eycleshymer's clearing fluid (equal parts of bergamot oil, cedar oil, and carbolic acid), which clears readily from 95 per cent. alcohol. Mount in balsam.

The following schedules for staining celloidin sections will give the student a start. The times given will vary with the thickness of the section and character of the tissue.

a. For staining in Delafield's hæmatoxylin and eosin:

1. Seventy per cent., 50 per cent., and 35 per cent. alcohol, 2 to 5 minutes each.
2. Delafield's hæmatoxylin, 5 to 30 minutes.
3. Wash in water, 5 minutes.
4. Thirty-five per cent. and 50 per cent. alcohol, 2 to 5 minutes each.
5. Acid alcohol (1 cc. hydrochloric acid + 100 cc. of 70 per cent. alcohol) until the stain is extracted from the celloidin, or at least until the celloidin retains only a faint pinkish color.
6. Seventy per cent. alcohol (not acid) until the purple color replaces the red due to the acid.
7. Eosin (preferably a 1 per cent. solution in 70 per cent. alcohol), 2 to 5 minutes.
8. Eighty-five per cent., 95 per cent. alcohol, 2 to 5 minutes each. Remember that 100 per cent. alcohol is not to be used with celloidin sections.

9. Eycleshymer's clearing fluid until transparent, usually 1 or 2 minutes, but sometimes 5 or 10.
10. Mount in balsam.

b. For staining in safranin and Delafield's hæmatoxylin:

1. Seventy per cent., 50 per cent., and 35 per cent. alcohol, 2 to 5 minutes each.
2. Safranin, 6 to 24 hours, preferably the longer period.
3. Acid alcohol (try about 1 drop of hydrochloric acid to 30 cc. of 70 per cent. alcohol) until the stain is removed from the celloidin or at least becomes very faint.
4. Fifty per cent. and 35 per cent. alcohol, 2 to 5 minutes each.
5. Delafield's hæmatoxylin, 2 to 5 minutes.
6. Wash in water, 5 minutes.
7. Thirty-five per cent. and 50 per cent. alcohol, 2 to 5 minutes each.
8. Acid alcohol (the same as was used for the safranin) until the stain is extracted from the celloidin. If it is found that this double immersion in acid alcohol extracts too much safranin, the third step may be omitted or shortened by removing the sections to the 50 per cent. alcohol before the stain is thoroughly removed from the celloidin.
9. Eighty-five per cent. and 95 per cent. alcohol, 2 to 5 minutes each.
10. Eycleshymer's clearing fluid until cleared.
11. Mount in balsam.

The celloidin method has its disadvantages as well as its advantages. It is extremely slow and tedious, and it is rarely possible to cut sections thinner than 10 μ, while, on the other hand, it gives smoother sections. The entire absence of heat makes it very useful for delicate, succulent tissues. Stems and roots which cannot be handled at all in paraffin cut well in celloidin, and much larger sections can be cut than in paraffin.

When material is to be imbedded, use celloidin as a last resort. Use paraffin when you *can*, celloidin when you *must*.

I am indebted to my friend Mr. W. B. MacCallum for several suggestions in regard to this method.

CHAPTER X.

THE GLYCERINE METHOD.

It is hard to get the filamentous algæ and fungi into balsam without shrinking; consequently, these forms are usually mounted in glycerine or glycerine jelly.

Flemming's fluid and chromo-acetic acid are good fixing agents. Corrosive sublimate in water, or in 70 per cent. alcohol, used hot, is also to be recommended. For general morphology, stain for six hours or over night in a ½ per cent. aqueous solution of eosin, transfer directly to a 1 per cent. solution of acetic acid in distilled water, and allow it to act for about five minutes; wash thoroughly in water to remove the acid, and then put the material into a watch-glass in a 10 per cent. solution of glycerine in water. The watch-glass should be kept as free from dust as possible, but should not be covered. As soon as the solution appears to be about as thick as pure glycerine, the material is ready for mounting. Place a small quantity of the material on a slide, arrange it carefully, add a small drop of glycerine, and a round cover. Seal with gold size (a varnish used by painters in laying gold leaf). None of the sealing media will stick to moist surfaces, hence it is essential that there should be only enough glycerine to come to the edge of the cover. If it is desired to mount rather large specimens, like the antheridia and oögonia of *Chara*, it is best to spin a ring on the slide, thus forming a shallow cell. Before the ring becomes hard the material may be mounted and sealed, or the ring may be allowed to harden, and just before a mount is to be made a very thin ring of the varnish may be added. The mount is firmer if the cover is not only sealed in the usual way, but also sticks to the ring underneath.

If glycerine jelly is to be used, place the bottle in warm

water until the jelly becomes liquid, but avoid any unnecessary heat. Take the material from the glycerine, add a drop of the warm jelly, and seal as before. More detailed directions for mounting material in glycerine are given in the chapters on algæ and fungi.

Part II.

In the preceding chapters the principles and methods of technique have been described in a general way. It is often difficult, especially for a beginner, to apply general principles to specific cases, and, besides, the types which he might select for the preparations might not form a symmetrical collection. Consequently, a series of forms has been selected which will not merely serve for practice in microscopical technique, but will also furnish the student with preparations for a fairly satisfactory study of plant structures from the algæ up to the angiosperms. It is not at all our purpose to discuss general morphology, but rather to answer, by means of sketches and specific directions, the multitudinous questions which confront the instructor in the laboratory. For those who have had a thorough training in general morphology the following suggestions will be in some degree surperfluous. Those who are beginning the study of minute plant structure are referred to the standard text-books for descriptions of the plants mentioned here.

CHAPTER XI.

THALLOPHYTES. ALGÆ.

CYANOPHYCEÆ.

Wasserblüthe.—These forms occur as scums, often iridescent, on the surface of stagnant or quiet water. Some of the commonest forms are *Cœlosphærium* and *Anabæna* (*fig. 13*). Some of the Chlorophyceæ also occur as *Wasserblüthe*. Where the material is very abundant, it may be collected by simply skimming it off with a wide-mouthed bottle, but where it is rather scarce, it is better to filter the water through a cloth, and finally rinse the algæ off into a bottle. Enough formalin may now be added to the water in the bottle to make a 2 per cent. solution. The material may be kept here indefinitely, but after a few hours it is ready for use. If the forms are small, like *Anabæna*, smear a slide lightly with Mayer's albumen fixative, as if for paraffin sections, add a drop of the material and allow it to dry, heat the slide gently to coagulate the albumen, or immerse the slide in strong alcohol for a few minutes, and then proceed with the staining. Cyanin and erythrosin is a good combination for differentiating the granules. Delafield's hæmatoxylin, used alone, stains some granules purple and others red. Iron alum-hæmatoxylin is excellent for heterocysts. If the forms are large enough to collapse with such treatment, the glycerine method may be employed.

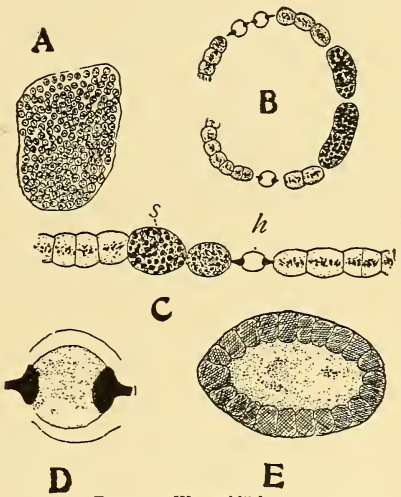

FIG. 13. Wasserblüthe.

A, Cœlosphærium Kuetzingianum. *B*, Anabæna flos-aquæ. *C*, Anabæna gigantea. *D* and *E*, a heterocyst and spore of A. gigantea drawn from paraffin sections stained in cyanin and erythrosin.

If it is desirable to make paraffin sections, put the material, drop by drop, on a piece of blotting paper until an appreciable layer of sediment is obtained. Get the paper with its material into paraffin by the usual method, taking great care not to wash the algæ off. After imbedding, trim away the paper and dip the block in melted paraffin. Sections can now be cut and stained in the usual manner.

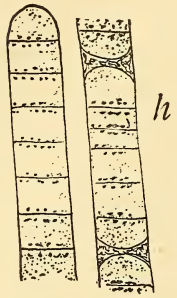

FIG. 14. Oscillaria.

Portions of two filaments, the one at the right showing a hormogonium, *h*.

Oscillaria.—For most purposes it is best to study *Oscillaria* in the living condition. It is readily found in watering troughs, in stagnant water, on damp earth, and in other habitats. The commonest forms have a deep blue-green or brownish color. For the purposes of identification and herbarium specimens, the material may simply be placed on a slip of mica and allowed to dry. When wanted for use, add a drop of water and a cover, and the mount is ready for examination. For sections or for glycerine mounts fix in chromo-acetic acid. (*Fig. 14.*)

Rivularia.—This form is readily found on the underside of the leaves of water-lilies (*Nuphar*, *Nymphæa*, etc.), but is also abundant on submerged leaves and stems of other plants. It occurs in the form of translucent, gelatinous nodules of various sizes. Chromo-acetic acid gives beautiful preparations, but good results can also be secured from formalin or picric-acid material.

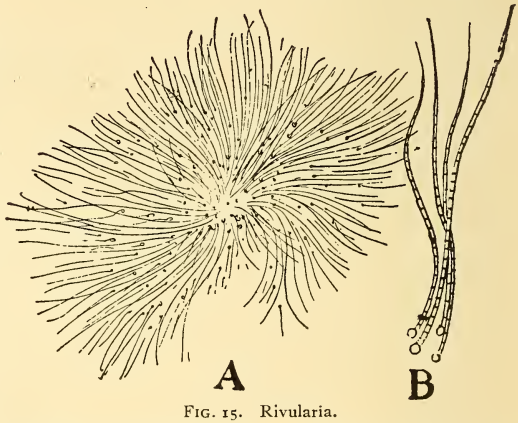

FIG. 15. Rivularia.

A, nodule crushed under cover-glass. *B*, four filaments more highly magnified, showing heterocysts at the base.

The most instructive preparations for morphological study

can be obtained by the glycerine method. Stain in eosin or Mayer's hæm-alum. When ready for mounting, crush a small nodule by pressing on the cover-glass. *Fig. 15* is drawn from such a preparation. The paraffin method is easily applied, since the gelatinous matrix keeps the plants in place. *Glœotrichia*, *Nostoc*, and forms of similar habit may be prepared in the same way.

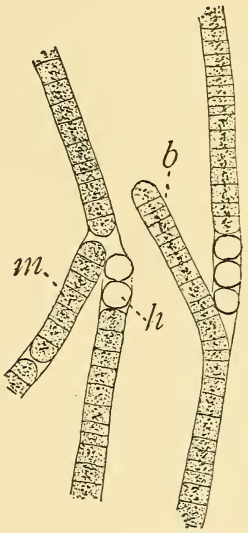

FIG. 16. Tolypothrix. *b*, a false branch. *h*, heterocysts.

Tolypothrix.—This form occurs as small tufts, either floating in stagnant water or attached to plants and stones. It furnishes an excellent example of false branching. (*Fig. 16.*) *Scytonema* is a similar form which is fairly common. The glycerine method should be employed for permanent preparations, but this, like all small filamentous algæ, may be dried on mica for herbarium purposes.

CHLOROPHYCEÆ.

The ponds, ditches, and rivers of any locality will yield an abundance and variety both of the unicellular and multicellular members of this group. The unicellular and filamentous members, together with such forms as *Volvox*, are best prepared by the glycerine method. The structure is so much more complicated than in the Cyanophyceæ that it demands far more care and skill to make good preparations. Chromo-acetic acid is a good killing and fixing agent for the whole group, but Flemming's fluid (weaker solution) seems to be a little better in some instances. Very good results have been obtained by adding about 5 cc. of 1 per cent. osmic acid to 100 cc. of chromo-acetic acid (Schaffner's formula). A formula which gives satisfactory results with *Spirogyra* may cause plasmolysis with *Cladophora*. The given filament should be placed under the microscope in the fixing agent, and, if plasmolysis occurs, the

chromic should be weakened or the acetic strengthened until the suitable proportions are determined. This is a slow process, but *Cladophora* and *Vaucheria* are almost sure to shrink without it. About twenty-four hours in any of the chromic series and a four to ten hours washing in water will be sufficient for members of this group. Only a few of the most familiar forms will be mentioned.

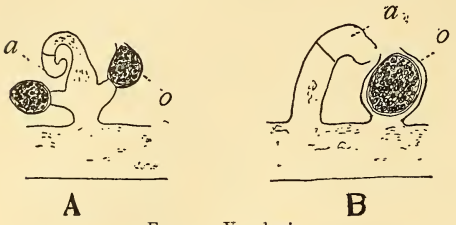

FIG. 17. Vaucheria.

A, Vaucheria geminata. *B*, V. sessilis. *a*, antheridia. *o*, oögonia.

Vaucheria.—This form can always be obtained in greenhouses, especially in the fernery, where it forms a green felt on the pots. The greenhouse form is likely to be *Vaucheria sessilis*. Another species, *V. geminata*, is very common in the spring, when it may be found in ponds and ditches. (*Fig. 17.*) It is extremely difficult to get mounts showing the nuclei. The following method is sometimes successful:

1. Chromo-acetic acid (Schaffner's formula), 24 hours.
2. Wash in water, 4 to 10 hours.
3. Iron alum, 2 to 4 hours.
4. Water, 15 to 30 minutes.
5. One-half per cent. hæmatoxylin, over night.
6. Wash in water, 5 to 10 minutes.
7. Iron alum until details become clear. This may take only a few minutes, but may take an hour.
8. Wash thoroughly in water, 1 to 4 hours.
9. Ten per cent. glycerine and allow the glycerine to thicken.
10. Mount and seal.

Cladophora.—This is found attached to sticks and stones in quiet or running water. It is easily recognized by its characteristic branching. (*Fig. 18.*) The nuclei of the cœnocytic segments are readily brought out by the method just described for *Vaucheria*. Alum carmine and Mayer's hæm-alum are also good stains for the nuclei.

Hydrodictyon.—This is popularly known as the "water-net." Nets of all sizes should be selected for study. The segments

are cœnocytic, and the nuclei are hard to differentiate except in the younger segments. The method given for *Cladophora* yields good results. The young nets forming within the older segments are easily demonstrated by Mayer's hæm-alum. Paraffin sections stained in cyanin and erythrosin, iron alum-hæmatoxylin, or the safranin-gentian violet-orange combination will repay the trouble, especially in case of the oldest segments within which new nets are forming. The habit is beautifully shown in preparations stained with eosin. The eosin (1 per cent. aqueous solution) should act for about twenty-four hours. Then transfer to 1 per cent. acetic acid for a few minutes. If the stain comes out rapidly in the acid, one minute may be sufficient, but if the stain does not wash out, it is better to let the acid act for four or five minutes. Wash thoroughly in water to remove all trace of acid, or the preparation will fade. Transfer to 10 per cent. glycerine and proceed as usual. (*Fig. 19.*)

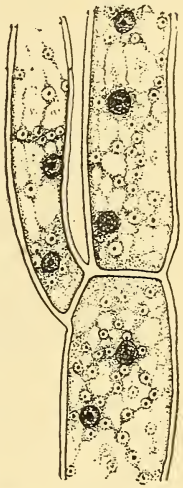

Fig. 18. Cladophora. Fixed in chromo-acetic acid, stained in Haidenhain's iron alum-hæmatoxylin.

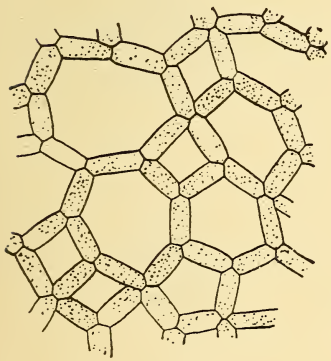

Fig. 19. Hydrodictyon. A small portion of a young net.

Spirogyra. — This is probably the most widely known of all the algæ, and, fortunately, it is rather easy to obtain beautiful and instructive preparations. The following is a good fixing agent for most *Spirogyras:*

Chromic acid, $\frac{4}{10}$ g.
Glacial acetic acid, $\frac{6}{10}$ g.
Water, 99 cc.

The addition of 1 cc. of 1 per cent. osmic acid seems to improve it without causing any blackening. Flemming's weaker solution is excellent. If it causes too much blackening, as it probably will, the material, after being washed, should be placed in weak peroxide of hydrogen (one part H_2O_2 to three parts H_2O) until the blackening due to osmic acid disappears. After

a moment's washing in water it is then ready for the stain. The iron alum-hæmatoxylin, as just described, brings out the nuclei and pyrenoids with great distinctness. (*Fig. 20.*) A few minutes in aqueous eosin after the last washing in water often gives a beautiful differentiation, but the preparations will be quite

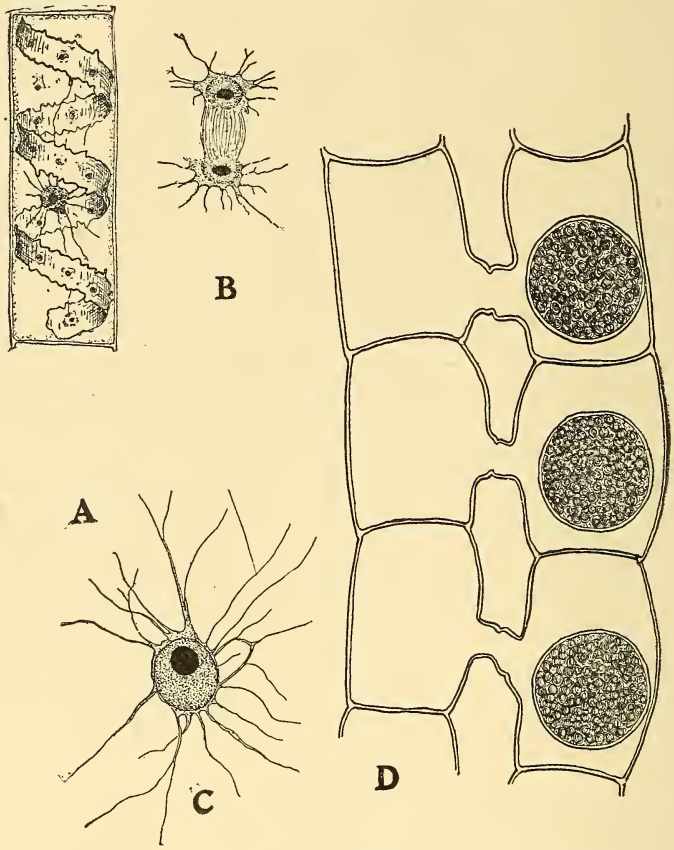

FIG. 20. Spirogyra.

From material fixed in chromo-acetic acid and stained in iron alum-hæmatoxylin. *A*, single cell showing nucleus, chromatophore, and pyrenoids. *B*, a nucleus undergoing division. *C*, a resting nucleus. *D*, zygospores, each showing two nuclei.

inferior if the eosin is allowed to stain too deeply. Mayer's hæm-alum is a better stain for stages in conjugation.

It is difficult to get *Spirogyra* into paraffin without shrinking, but it can be done. Watch carefully and note where plasmolysis

occurs. There will probably be little or no trouble until the transfer from 100 per cent. alcohol to the clearing agent. Make this transfer as gradual as may be necessary. After the pure xylol or other clearing agent is reached, add a lump of paraffin large enough to saturate the clearing fluid at a temperature of 40 to 45° C. Allow the xylol to evaporate at this temperature and imbed as usual, taking care to keep the filaments as nearly parallel as possible. Many elegant combinations, like cyanin and erythrosin, fuchsin and iodine green, safranin-gentian violet-orange, and others not available for glycerine preparations, can be used with paraffin sections. It is comparatively easy to get any such alga into celloidin. Safranin and Delafield's hæmatoxylin then make a good combination.

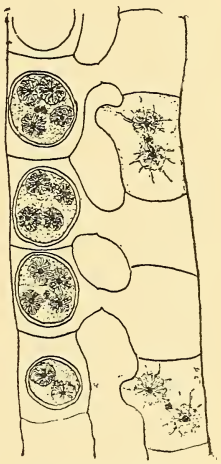

Fig. 21. Zygnema.

The filament at the left shows three zygospores and one parthenogenetic spore which is distinguished by having only two chromatophores. The filament on the right shows two cells, each with a pair of stellate chromatophores. Drawn from material fixed in 2 per cent. formalin and stained in iron alum-hæmatoxylin.

Zygnema — Use the same methods as for *Spirogyra*. In staining conjugating material the stain should be extracted until the four chromatophores of the zygospore become distinct. The nuclei are comparatively small and unsatisfactory. The stellate chromatophores are well brought out by alum carmine. If iron alum-hæmatoxylin and eosin are used, the eosin may well be much deeper than in case of *Spirogyra*. (*Fig. 21.*)

Diatoms.— Diatoms and desmids have been variously classified, and their position is not yet fully determined. Living diatoms are often found clinging in great numbers to filamentous algæ, or forming gelatinous masses on various submerged plants. It is difficult to get really good preparations showing the nucleus and chromatophores. If the diatoms are clinging to filamentous algæ, the algæ with the diatoms attached may be put into chromo-acetic acid (twenty-four hours), washed in water, stained, passed up through the alcohols, and cleared in xylol, or, better, in clove oil or bergamot oil, which do not dry up so

rapidly. Here the diatoms may be picked or scraped off from the other algæ, which will probably have become much shrunken by this treatment. Mount in balsam. Haidenhain's iron alum-hæmatoxylin is recommended for the nucleus and the centrosome, which is quite prominent in diatoms. Delafield's hæmatoxylin and erythrosin give a good view of the nucleus and chromatophore. If a glycerine mount is preferred, the iron alum-hæmatoxylin is a good stain.

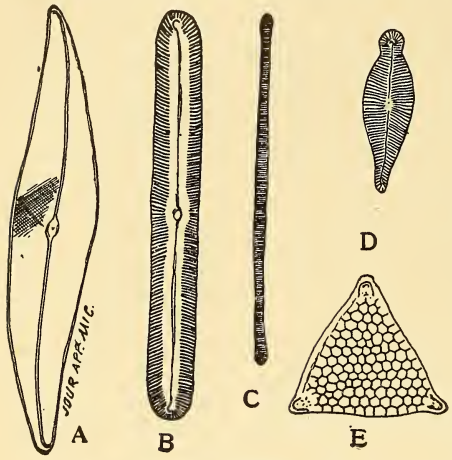

FIG. 22. Diatoms. × 255.

A, Pleurosigma angulatum. *B*, Navicula dactylis. *C*, Synedra biceps. *D*, Gomphonema sphærophorum. *E*, Triceratium sp.

When the material is in gelatinous masses, it may be fixed in chromo-acetic acid and imbedded in paraffin. There will, of course, be some difficulty in cutting, and many frustules will be broken, but there will, nevertheless, be occasional views which show details better than when the diatoms are mounted whole.

The silicious shells of diatoms are among the most beautiful objects which could be examined with the microscope (*fig. 22*). To obtain perfectly clean mounts requires considerable time and patience, but when the material is once cleaned, preparations may be made at any time with very little trouble. Diatom enthusiasts have devised numerous methods for cleaning diatoms, and separating the various forms from each other, but we shall give here only a few simple, practical methods.

Material for mounts of frustules of living forms "may be obtained by skimming off the brownish scum found on ponds, by squeezing out water weeds, by scraping sticks and stones which are covered at high water, or from the mud of filter beds at pumping works, or in other places. The material is put in a dish of water, and after it has settled the water is decanted.

This is repeated until the water will clear in about one-half hour. The sediment is then treated with an equal bulk of sulphuric acid, after which bichromate of potash is added until all action ceases. After a couple of hours the acid is washed out. To separate the diatoms, place the sediment in a glass dish with water, and when the water becomes clear give the dish a slight rotary motion. This will bring the diatoms to the top, when they may be removed with a pipette and placed in alcohol. To mount, place a number in distilled water, evaporate a few drops of the mixture on a cover-glass, which is then mounted on a slide in balsam." (From a review of Dr. Wood's paper on "Diatoms," *Jour. App. Mic.*, March, 1899.)

Many scouring soaps and silver polishes contain large quantities of fossil diatoms, and the diatomaceous earths are particularly rich. Break up a small lump of such material and boil it in hydrochloric acid. A test-tube is very convenient for this process. Let the diatoms settle, pour off the acid, and then wash in water. As soon as the diatoms settle, the water should be poured off. The washing should be continued until the hydrochloric acid has been removed. When the washing is complete, pour on a little absolute alcohol, and after a few minutes pour off the alcohol and add equal parts of turpentine and carbolic acid. The material will keep indefinitely in this condition and may be mounted in balsam at any time. In making a mount, put a little of the material on a slide and allow it to become dry, or nearly dry, and then add the balsam and cover. If the balsam should be added too soon, the diatoms are likely to move to the edge of the cover.

Desmids.—When these forms are very abundant, they may be treated like the filamentous algæ, except that extreme care must be taken lest the desmids be lost while changing fluids. It often happens that a single desirable desmid appears when examining field collections. In such a case, remove it with a fine pipette, and get it into a drop of water on a clean slide, invert it over a bottle of 1 per cent. osmic acid for a minute, leave the slide exposed to the air until the water has almost all

evaporated, and then add a drop of 10 per cent. glycerine. In a few hours (six to twenty-four) put on a cover and seal. It requires more time, care, and patience than it is worth to attempt staining in such a case. (*Fig. 23.*)

Oedogonium.—In selecting material it will be better for teaching purposes to choose the larger monœcious forms. The nuclei, pyrenoids, and chromatophores are easily differentiated. Mayer's hæmalum is a good stain, especially for the antheridia. Alum carmine or eosin will bring out the "caps." (*Fig. 24.*)

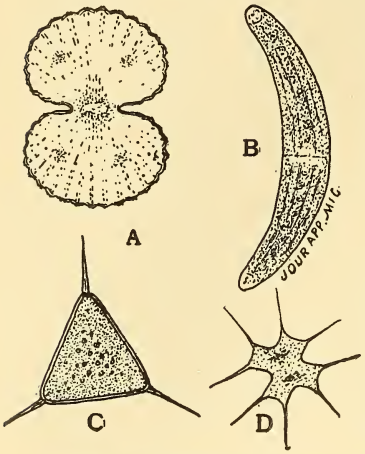

FIG. 23. Desmids. × 255.
From glycerine preparations. Not stained. *A*, Cosmarium pectinoides. *B*, Closterium cucumis. *C*, Staurastrum cornutum. *D*, Arthrodesmus octocornis.

Chara.—This form is so large and coarse that it hardly pays to mount it in glycerine. If a glycerine mount is desired to show the antheridia and oögonia in position, spin a ring of cement on the slide, thus making a cell in which small portions of the plant may be mounted. For paraffin sections select the tip of the plant, a piece about half an inch in length. Sections of this may show, not only the large apical cell, but also various stages in the development of antheridia and oögonia. Delafield's hæmatoxylin is a very good stain for the apical cell and for the development of antheridia and oögonia. The later stages in the development of antherozoids are brought out more clearly by the safranin-gentian violet-orange, or by cyanin and erythrosin, but here unusual care must be taken not to stain too deeply.

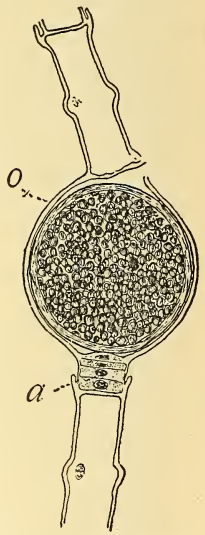

FIG. 24. Oedogonium nodulosum.
a, antheridia. *o*, oögonium. Drawn from material fixed in 1 per cent. chromic acid, and stained in Mayer's hæm-alum.

Good preparations showing shield, manubrium, capitula, and filaments may be obtained by staining in bulk in alum carmine

and then crushing the antheridium under the cover-glass after the specimen is in balsam. (*Fig. 25.*)

PHÆOPHYCEÆ.

The brown algæ are almost exclusively marine. The slime, so prevalent in the group, often makes the technique difficult.

Ectocarpus.— Fix in chromo-acetic acid (twenty-four hours), wash in fresh water, since the salt of sea water may cause inconvenience in subsequent processes. Stock material should be passed up to 70 per cent. alcohol for safe keeping. Eosin or Mayer's hæm-alum are good for glycerine mounts. If paraffin sections are to be made, the material must be brought very gradually from absolute alcohol into the clearing agent, and from the clearing agent into the paraffin. (*Fig. 26.*)

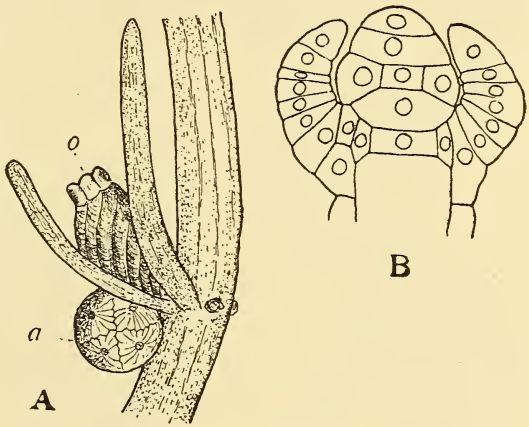

FIG. 25. Chara.

A, portion of a branch showing an antheridium, *a*, and an oögonium, *o*. *B*, median longitudinal section of an apical cell. Drawn from a preparation fixed in chromo-acetic acid, and stained in Delafield's hæmatoxylin.

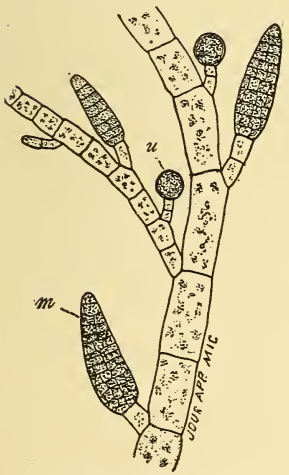

FIG. 26. Ectocarpus confervoides.

From a preparation stained in Mayer's hæm-alum, and mounted in glycerine. × 255. *m*, multilocular sporangium. *u*, unilocular sporangium.

Other filamentous members of the group, as well as the more delicate membranous forms, may be treated like *Ectocarpus*.

Fucus.— *Fucus* may be fixed, washed, and preserved like *Ectocarpus*. It is difficult to get paraffin sections across the whole fertile branch, but elegant sections may be obtained by cutting

narrow strips containing a few conceptacles. The safranin-gentian violet-orange combination is good for such sections. For such views as are represented in *fig. 27*, *C* and *D*, the material should be stained in bulk in borax carmine or alum carmine. The process for borax carmine is as follows:

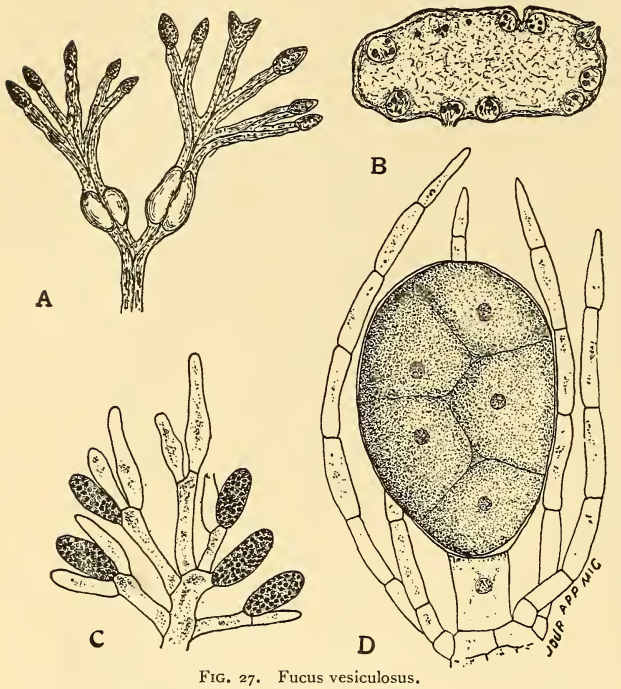

FIG. 27. Fucus vesiculosus.

A, small portion of plant showing bladders and fruiting branches. One-half natural size. *B*, transverse section of fruiting branch showing oögonial conceptacles. × 6. *C*, antheridia and paraphyses. From a preparation fixed in chromo-acetic acid, stained in borax carmine, teased out and mounted in balsam. × 255. *D*, oögonium showing five of the eight oöspheres. Prepared as in *C*.

1. Borax carmine, 24 hours.
2. Acid alcohol (2 drops of HCl in 50 cc. of 70 per cent. alcohol), until the color becomes a clear red. This may take an hour or even a day.
3. Seventy to 100 per cent. alcohol, 2 hours each.
4. Clear in cedar oil, bergamot oil, or oil of cloves.
5. Tease out the contents of the conceptacles sufficiently to show details, and mount in balsam.

The process for alum carmine is the same, except that no acid alcohol is used.

Sections like that shown in *B* are easily cut in celloidin. After staining in borax carmine or alum carmine, imbed in celloidin in the usual way. After hardening the celloidin in chloroform, put the block into 95 per cent. alcohol for two or three hours, and then into Eycleshymer's clearing fluid (equal parts bergamot oil, cedar oil, and carbolic acid), until thoroughly cleared. The block may be left here indefinitely, and sections

may at any time be mounted in balsam as soon as they are cut.

Chorda, *Laminaria*, and similar forms may be treated like *Fucus*.

RHODOPHYCEÆ.

The red algæ belong almost exclusively to salt water, but a few genera are found only in fresh water, usually in running water, and a few forms occur both in salt and in fresh water.

The technique is more difficult than in the case of the brown algæ. Until something better is suggested, the same method of fixing and washing may be used as for the brown algæ. Picric acid, corrosive sublimate, and absolute alcohol have been tried, but do not give as good results as the chromo-acetic acid or Flemming's fluid.

Batrachospermum.—This is a green, fresh-water member of the red algæ. It is not very uncommon in small streams. (*Fig. 28.*)

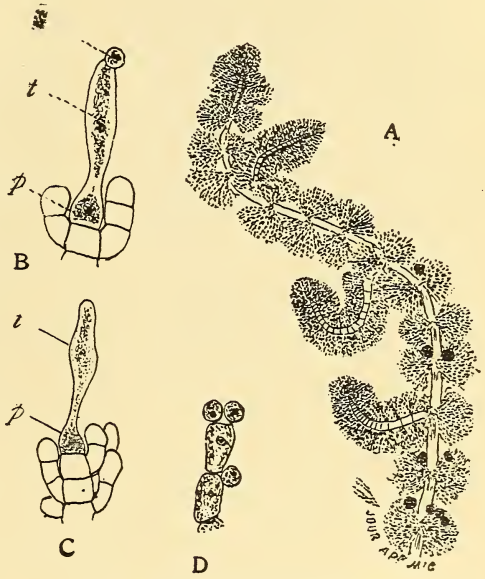

FIG. 28. Batrachospermum moniliforme.

From a preparation stained in Mayer's hæm-alum and mounted in glycerine. *A*, portion of plant showing branches and several cystocarps. × 25. *B*, a procarpic branch showing carpogonium (*p*), and trichogyne (*t*), with an antherozoid (*s*) attached. × 255. *C*, a younger branch showing carpogonium and trichogyne. × 255. *D*, branch with three antherozoids. × 255.

The cells are so small that it is hardly worth while to attempt sectioning them. Very good preparations showing the nuclei may be obtained by staining in Mayer's hæm-alum, or Haidenhain's iron alum-hæmatoxylin. After the material is in glycerine ready for mounting, tease out a small portion, and still further dissociate the filaments by tapping smartly on the cover.

Material stained in eosin shows the external structure well, but may not bring out the nuclei.

Polysiphonia.— For preparations like those shown in *fig. 29* eosin is a very good stain. To get a brilliant coloring, stain for about twenty-four hours, so that the 1 per cent. acetic acid may be allowed to act for several minutes without making the stain look weak. Wash thoroughly in water. Not the slightest trace of color should be allowed to come out in the glycerine.

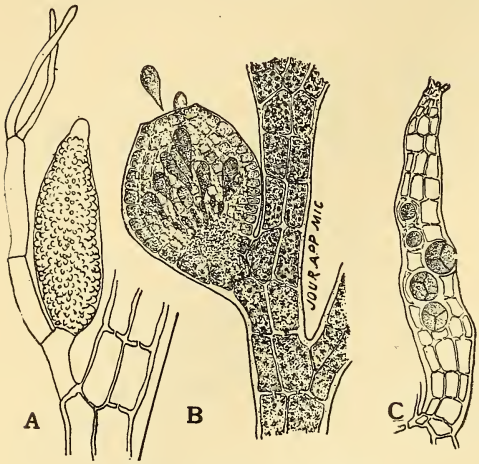

FIG. 29. Polysiphonia fibrillosa.

From a preparation fixed in chromo-acetic acid, stained in eosin, and mounted in glycerine. × 255. *A*, an antheridium. *B*, a cystocarp with carpospores. *C*, tetrasporic branch with tetraspores.

Sections showing the central and peripheral siphons and other gross features are easily cut in celloidin. It is not very difficult to cut paraffin sections, but the nuclei are so small and so hard to bring out that such preparations had better be left for the specialist.

CHAPTER XII.

THALLOPHYTES. FUNGI.

SCHIZOMYCETES.

Bacteria.—The methods of modern bacteriological technique are so numerous and so specialized that we must refer to laboratory manuals for instruction in this subject. The method given here will merely enable the student to study the form and size of those bacteria which are more easily demonstrated.

Foul water at the outlets of sewers and such places will usually afford an abundance of coccus, bacillus, spirillum, and beggiatoa forms. Place a drop of the water on a slide, heat it gently until the water evaporates, then stain with fuchsin or methyl violet, dehydrate, clear in xylol, and mount in balsam (*fig. 30*).

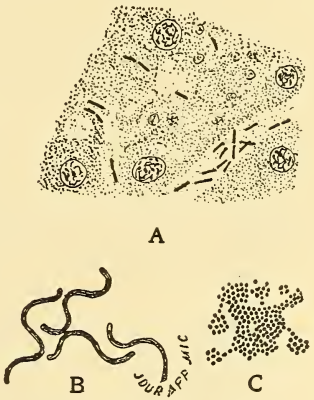

FIG. 30. Bacteria. ×535.

A, Bacillus anthracis, from a paraffin section cut from the liver of a mouse. Fixed in chromo-acetic acid, stained in methyl violet and bismark brown, and mounted in balsam. *B*, Staphylococcus pyogenes aureus. From a preparation stained in gentian violet. *C*. Spirillum sp. From a preparation stained in fuchsin.

Fine preparations may be obtained by inoculating a mouse with *Anthrax* or some other form, and then cutting paraffin sections of favorable organs. Gentian violet with a faint bismark brown for a background makes a good combination. The following schedule gives good results with Anthrax and many other bacteria:

1. Gentian violet, 5 minutes.
2. Rinse in water a few seconds.
3. Gram's solution (iodine 1 gram, potassium iodide 2 grams, water 300 cc.) until the color is almost or quite black; this will generally require 1 or 2 minutes.
4. Ninety-five per cent. alcohol until the color has nearly disappeared.

5. Rinse in water and examine. If the bacteria are well stained, a counter-stain for the background may be added.
6. Erythrosin, 3 or 4 seconds; or bismark brown 5 or 10 seconds.
7. Ninety-five and 100 per cent. alcohol, dehydrating as rapidly as possible. Not more than 5 or 10 seconds can usually be allowed.
8. Xylol.
9. Balsam.

Leptothrix may often be obtained by scraping the inside of the cheek. *Beggiatoa*, a form with oscillating movements like *Oscillaria*, is often found in foul water. Its presence may be indicated by whitish patches on the bottom.

It is doubtful whether the bacteria possess even a morphological forerunner of the nucleus of higher plants, consequently there need be no disappointment if the larger forms, like some of the Beggiatoas, fail to show a nucleus.

MYXOMYCETES.

With the exception of a few forms like *Fuligo* (often found on oak stumps and on oak bark in tan yards), the Myxomycetes are small, and are usually overlooked by collectors. (*Fig. 31.*) A careful examination of rotting logs in moist woods will usually reveal an abundance of these delicate and beautiful organisms. They should be pinned to the bottom of a box for safe carrying. For herbarium specimens they are simply allowed to dry, and are then fastened with glue or paste to the bottom of a small box.

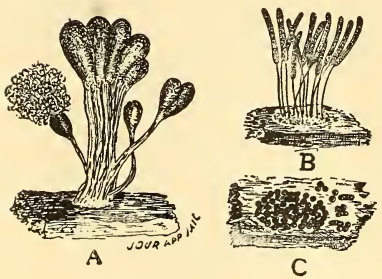

FIG. 31. Myxomycetes.

Growing on rotten wood. *A*, Hemitrichia rubiformis. × 20. *B*, Stemonitis ferruginea. Natural size. *C*, Trichia varia. × 1½.

Plasmodia and young sporangia may be fixed in chromo-acetic acid or Flemming's fluid. Sections are easily cut in paraffin, and should not be more than 5 μ in thickness, and should be thinner, if possible. Acid fuchsin and iodine green is a good stain. Delafield's hæmatoxylin used alone or with a little orange G. is also to be recommended. Excellent methods for

living cultures were given in the January and February (1898) numbers of the *Journal of Applied Microscopy*.

PHYCOMYCETES.

Mucor.—This familiar mold appears with great regularity on bread. The following is a sure and rapid method for obtaining *Mucor:* Place a glass tumbler in a plate of water, put on the tumbler a slice of bread which has been exposed to the air for a day, and cover with a glass jar.

To obtain such a series as is shown in *A–D* of *fig. 32*, the material should be studied before the sporangia begin to turn black. The phase in the life-history indicated in *F–H* is rarely seen. The writer would be glad to hear from any who have met this phase, especially if the information could be accompanied by a few zygospores.

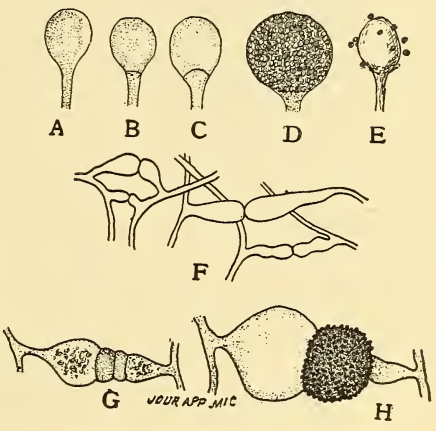

FIG. 32. Mucor stolonifer. × 255.

A–D, successive stages in the development of the sporangium. Drawn from living material. *E*, columella with a few spores adhering. *F–H*, stages in the formation of the zygospore. From a preparation fixed in corrosive sublimate, stained in Delafield's hæmatoxylin, and mounted in glycerine.

Corrosive sublimate (4 per cent.) in 50 per cent. alcohol, used hot, may be recommended as a fixing agent. Hæm-alum, or Delafield's hæmatoxylin, is good for glycerine preparations. Do not stain too deeply. A very satisfactory study may be made from the living material.

Cystopus.—This fungus is quite common on Cruciferæ, where the white "blisters" or "white rust" form quite conspicuous patches.

Affected portions of leaves and stems should be fixed in chromo-acetic acid and cut in paraffin. Sections 5 μ or less in thickness will be found most satisfactory. Safranin-gentian violet-orange seems to be the best stain for differentiating the nuclei. (*Fig. 33.*)

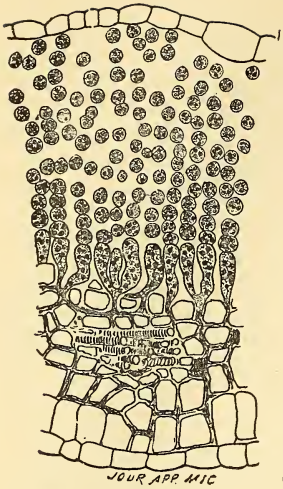

FIG. 33. Cystopus candidus on Capsella.

Trans. sec. of a blister on the leaf. × 255. From a preparation fixed in Flemming's fluid and stained in safranin-gentian violet-orange.

It is more difficult to get good sections of the plant in the oösporic condition. The oösporic phase of *Cystopus bliti* is easily recognized on *Amarantus*, where the oöspores may be seen with the naked eye by holding the leaf up to the light. While better nuclear staining can be secured with chromic or Flemming material, it will be found somewhat easier to cut material which has been fixed in picric acid (1 per cent. solution in 70 per cent. alcohol). Celloidin sections, stained in Delafield's hæmatoxylin, can be recommended for showing the position of oögonia and antheridia, although such sections are too thick to give satisfactory views of the nuclei.

ASCOMYCETES.

This group, popularly known as the "sac fungi," contains an immense number of saprophytic and parasitic forms. Yeast, green mold on cheese and leather, leaf curl of peach, black knot of cherry and plum, and the powdery mildews are familiar to everyone. The few objects selected will enable the student to experiment, but he must not be discouraged if success does not crown the first attempt, for the group presents many difficulties.

Saccharomyces.—Until somewhat recently it was considered rather difficult to demonstrate the nucleus of the yeast cell. With fresh growing yeast the following method by Wager should be successful: Fix in a saturated aqueous solution of corrosive sublimate for at least twelve hours. Wash successively in water, 30 per cent. alcohol, 70 per cent. alcohol, and methyl alcohol. Place a few drops of alcohol containing the cells on a cover, and when nearly dry add a drop of water. After the yeast cells settle, drain off the water and allow the

cells to dry up completely. Place the cover, or slide, with its layer of cells in water for a few seconds, and then stain with a mixture of fuchsin and methyl green, or fuchsin and methylen blue. Mount in glycerine or in balsam.

Eurotium.— For class use or for permanent preparations it is best to select rather young material which shows various stages in development, from the swollen end of the hypha to the ripe spore (*fig. 34*). The nuclei are exceedingly small, and can hardly be demonstrated with eosin. Iron alum-hæmatoxylin would be better.

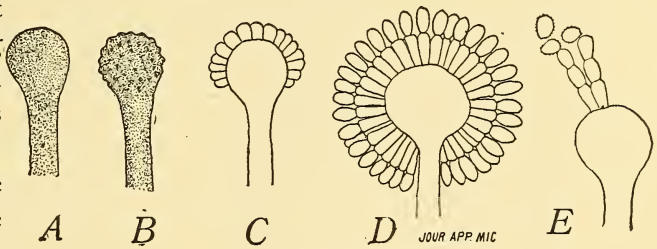

FIG. 34. Eurotium.

From material growing on a hectograph pad. Fixed in chromo-acetic acid, stained in eosin, and mounted in glycerine. *A–E*, successive stages in development. × 375.

The following schedule will give good mounts for habit study:

1. Fix in a saturated solution of corrosive sublimate in 50 per cent. alcohol. The addition of 1 cc. of glacial acetic acid to 100 cc. of this solution improves it. Use it hot.
2. After it cools, transfer to 50 per cent. alcohol and add, a few drops at a time, the iodine solution which is used in testing for starch. At first the brownish color caused by the iodine will disappear, but after a certain amount has been added the brown color will remain. The material is then ready for the next step.
3. Thirty-five per cent. alcohol, 5 minutes.
4. Eosin (aqueous), at least 5 minutes; a day will do no harm.
5. Put the material into 1 per cent. acetic acid for 15 seconds to 2 minutes. The material should still have a vivid red color when taken from the acid.
6. Wash in water. Use a considerable volume of water or change the water several times. If the acid is not all washed out, the preparations will fade.
7. Ten per cent. glycerine, and allow the glycerine to thicken.
8. Mount in glycerine or glycerine jelly, and seal.

Any of the filamentous fungi—like *Mucor*, *Thamnidium*, *Peronospora*, *Penicillium*, *Pythium*, *Saprolegnia*, etc.—can be mounted in this way. *Saprolegnia*, however, is much more satisfactory if stained in iron alum-hæmatoxylin.

A very rapid method for the unicellular and filamentous forms may be added:

1. One hundred per cent. alcohol, 2 minutes.
2. Eosin (aqueous), 2 minutes.
3. One per cent. acetic acid, 2 to 10 seconds.
4. Wash in water 5 minutes, changing frequently.
5. Mount directly in 50 per cent. glycerine, and seal.

If the material gets through the first four stages without shrinking, but collapses at the fifth, put it into 10 per cent. glycerine and allow it to thicken as usual.

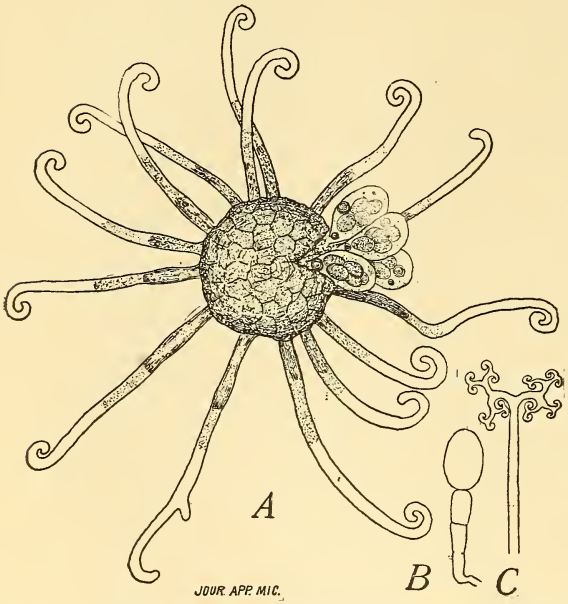

FIG. 35. *A*, Uncinula necator on Ampelopsis quinquefolia. × 192. Four asci containing ascospores have been forced out by pressing out the cover. Fixed in hot corrosive sublimate, stained in fuchsin, and mounted in balsam. *B*, a conidiospore; and *C*, an appendage of Microsphæra alni, drawn from living material. × 192.

Uncinula necator.— The mildews are found throughout the summer and autumn on the leaves of various plants. The lilac mildew (*Microsphæra alni*) and the mildew on the Virginia creeper (*Uncinula necator*) are particularly abundant. For herbarium purposes they may be preserved by simply drying the leaves under light pressure. When mounted for examination, the leaf should be soaked in water for a few minutes, after which the perithecia may be scraped off and mounted in water. The asci may then be forced out by pressing smartly on the cover. (*Fig. 35.*)

For permanent mounts of entire perithecia with appendages, fix in 3 per cent. formalin twenty-four hours, wash in water one hour, stain in aqueous eosin twenty-four hours, treat with 1 per

cent. acetic acid one minute, wash thoroughly in water, and then transfer to 10 per cent. glycerine, which should be allowed to concentrate as usual. If chromic acid, corrosive sublimate, or alcohol be used for fixing, the appendages become brittle, and very easily break off. However, the chromo-acetic mixtures are better if it is desired to make paraffin sections showing the development of the perithecium with its asci and spores. For this purpose the omnipresent *Erysiphe commune* on *Polygonum aviculare* is exceptionally favorable, because, after the material is fixed and in alcohol, the whole mycelium, with the developing perithecia, may be stripped from the leaf without the slightest difficulty, thus avoiding the necessity of cutting the leaf in order to get the fungus. The safranin-gentian violet-orange combination seems to give the best results, although cyanin and erythrosin are quite satisfactory when the stains are properly balanced.

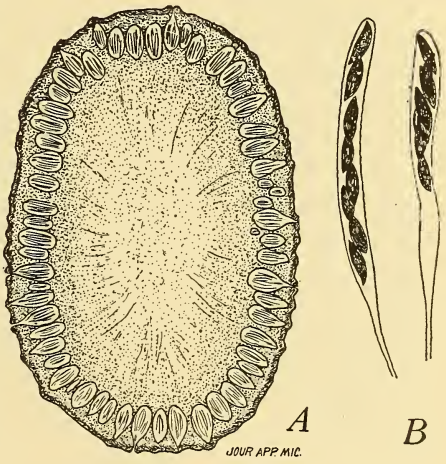

FIG. 36. Xylaria.

A, transverse section of a young stroma showing perithecia. × 8. Fixed in chromo-acetic acid, stained in bulk in alum carmine, imbedded in celloidin, and mounted in balsam. *B*, two asci with spores. × 245. The mature stroma was soaked for several days in equal parts of 95 per cent. alcohol and glycerine, and then imbedded in celloidin. Not stained.

Xylaria.—Many forms, like *Xylaria*, *Ustilina*, *Hypoxylon*, and *Nummularia*, in their mature condition, are woody and so extremely brittle that it is almost impossible to cut them. As good a plan as any seems to be to cut sections of the stroma about one-eighth of an inch thick, soak them in equal parts of glycerine and 95 per cent. alcohol, and then imbed them in celloidin in the usual way. They might be cut without imbedding, but most of the asci and spores would then be lost.

The younger stages, showing the development of perithecia and asci, are more interesting, and can be cut in paraffin and stained with ease (*fig. 36*).

Peziza.—The Pezizas and related forms are fleshy, and present but little difficulty in fixing, cutting, or staining. They are abundant in moist places, on decaying wood, or on the ground. The apothecia have the form of little cups, which are sometimes black and sometimes flesh-colored, but often orange, red, or green. For the development of ascospores in the ascus, Flemming's fluid (weaker solution), followed by safranin-gentian violet-orange, has given the best results with thin sections where the mitotic figures are to be studied. Cyanin and erythrosin is also to be recommended. Such sections should not be more than 5 μ in thickness. For a general morphological preparation, such as is shown in the figure, it is better to stain in bulk in alum carmine or in Delafield's hæmatoxylin, and then tease out the asci in glycerine or balsam. Sections thick enough to show the entire ascus are not usually as satisfactory as such teased preparations. (*Fig. 37.*)

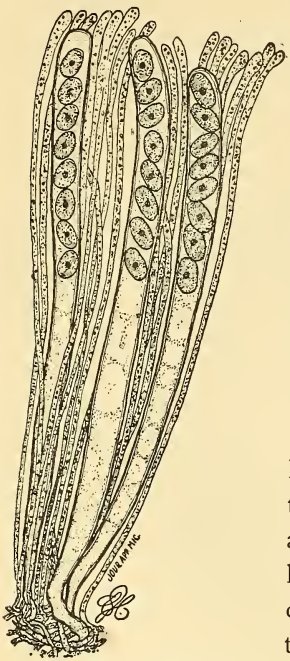

FIG. 37. Peziza odorata. Three asci and many paraphyses. × 245. Fixed in corrosive sublimate, stained in bulk in alum carmine. Teased out and mounted in balsam.

ÆCIDIOMYCETES.

The Æcidiomycetes comprise the rusts (*Uredineæ*) and the smuts (*Ustilagineæ*).

Puccinia graminis.—The common rusts of wheat and oats are familiar to everyone. The uredospores, or summer spores, known as the red rust, and the teleutospores (last spores), or winter spores, known as the black rust, are found in unfortunate adundance, but the æcidium stage on the barberry is not necessary for the vigorous development of rust in the United States, and is seldom found. Most teachers are obliged to depend upon botanical supply companies for this material. There are, however, various æcidia which are as good, or even better, for

morphological study. The æcidia growing on *Euphorbia maculata* (spotted spurge), and on *Arisæma triphyllum* (Jack-in-the-pulpit) are much easier to cut, and seem easier to stain. Delafield's hæmatoxylin, followed by a very light stain in erythrosin, is

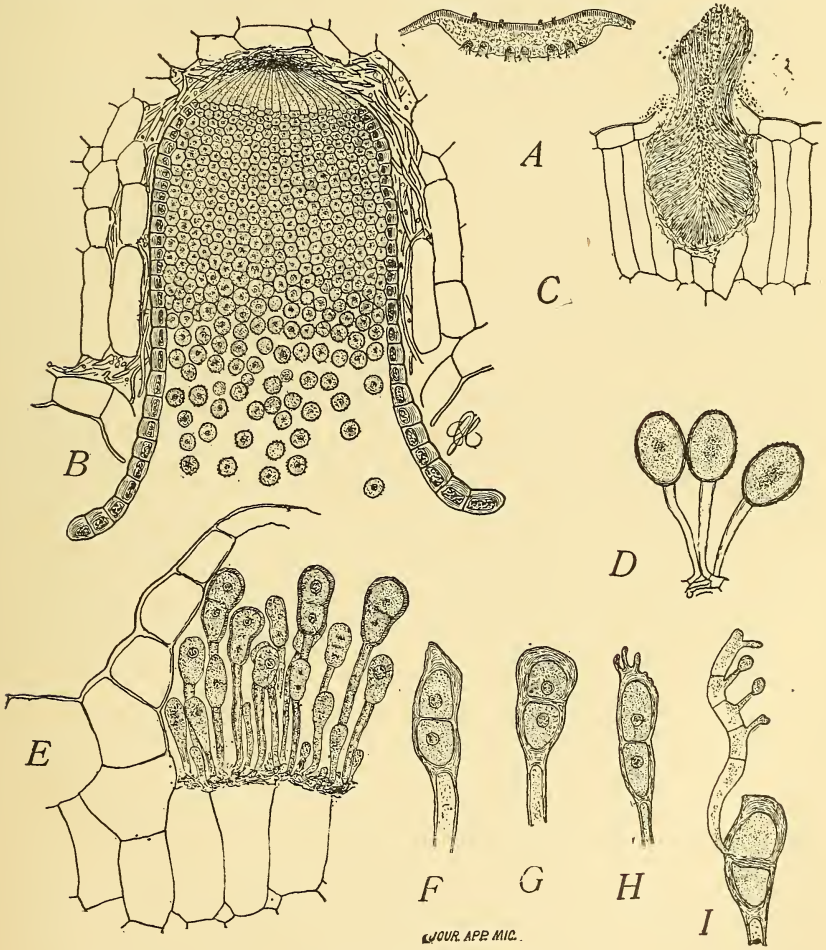

FIG. 38. Puccinia graminis.

A, transverse section of barberry leaf showing æcidia and spermagonia. × 7. *B*, longitudinal section of a single æcidium. × 192. Fixed in Flemming's weaker solution and stained in Delafield's hæmatoxylin. *C*, a single spermagonium. × 192. Fixed and stained as in *B*. *D*, three uredospores growing on oats. × 375. Fixed in 2 per cent. formalin, stained in bulk in alum carmine, and teased out in glycerine. *E*, section of young teleutospores on oats. × 375. Fixed in picro-acetic acid and stained in cyanin and erythrosin. *G*, *F*, *H*, three ripe teleutospores from a leaf of oats showing variation in form. × 375. *I*, germinating teleutospores. × 375.

good for both æcidia and spermagonia, especially after Flemming's fluid. It is rather difficult to get good sections of uredospores and teleutospores, because the leaves of wheat and oats are refractory objects to cut. The cutting is easier after picro-acetic acid than after corrosive sublimate or the chromic-acid series. (*Fig. 38.*)

Every class which studies the rusts should attempt to germinate the uredospores and teleutospores. For this purpose the hanging drop culture may be employed. Cement a rubber or zinc ring to the slide, or simply smear the lower surface of the ring with vaseline and press it tightly against the slide; smear the upper surface of the ring with vaseline, and over it invert the cover-glass with a shallow drop of water containing the spores (*fig. 39*). The uredospores germinate readily all summer, but it is said that the teleutospores will germinate only in the spring following their maturity. However, the teleutospores of many species, like *Puccinia xanthii* on *Xanthium canadense* (cocklebur), will germinate as soon as they ripen and will serve equally well for study. If a particularly good specimen is secured, it may be preserved by the method previously described for desmids, except that in this case it might be worth while to attempt staining with Mayer's hæm-alum, or with eosin.

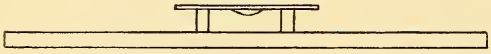

Fig. 39.

The smuts may be studied in the living material. The following method, recently described by Ellis, is worth remembering: A supply of smutted barley may be obtained by sowing soaked, skinned barley that has been plentifully covered by *Ustilago* spores. In such material it is easy to trace stages in the development of spores. Free-hand sections of ears about three-eighths of an inch long show the mycelium and spore clusters. If smutted ears be removed and kept floating on the water, the spores continue to develop and often germinate. For paraffin sections desirable stages should be fixed in Flemming's fluid or picro-acetic acid. Delafield's hæmatoxylin, followed by

a very light touch of erythrosin or acid fuchsin, will give a good stain.

For a study of the germinating spores and conidia, cultures may be made in beerwort on the slide or in watch crystals. Harper's method of making preparations from such material is ingenious and will undoubtedly prove valuable in making mounts of various small plant and animal forms. A drop of the material is taken up with a capillary tube and is then gently blown out into a drop of Flemming's weaker solution (fifteen minutes or an hour was sufficient for the fungus spores). Cover a slide with albumen fixative, as if for sections. A drop of the material, without previous washing, is drawn up into the capillary tube and touched lightly and quickly to the surface of the albumen. A series of such drops, almost as small as the stippled dots in a drawing, may be applied to the slide. The fixing agent may now be allowed to evaporate somewhat, but the preparation must not be allowed to dry. As the slide is passed rapidly through the alcohols, the albumen is coagulated, and the preparation may be treated just as if one were dealing with ribbons of sections.

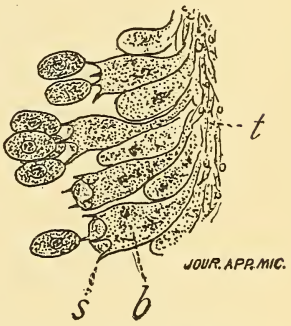

FIG. 40. Coprinus comatus.

Transverse section of a portion of one of the gills showing a part of the trama, *t*, and several basidia, *b*, each with four sterigmata, *s*. Spores have fallen off from many of the sterigmata. × 750.

BASIDIOMYCETES.

This is an immense group, of which the mushrooms, toadstools, puffballs, and bracket fungi are the most widely known representatives.

Coprinus comatus.—This is the common shaggy-mane mushroom. Cut from the cap pieces about one-fourth of an inch square, and fix in chromo-acetic acid or in Flemming's fluid. Portions in which the gills have just begun to turn brown will show the spores still attached to the sterigmata (*fig. 40*). If the gills have become dark brown or black, the spores will wash off before the sections can be mounted. Look in portions in which the gills are still white or only slightly changing color for the develop-

ment of basidia and spores. The nuclei, although rather small, are brought out nicely by safranin-gentian violet-orange. The same procedure may be observed for other forms of similar consistency, like many members of the genera *Boletus*, *Hydnum*, *Polyporus*, *Lycoperdon*, etc. Leathery or woody forms like *Stereum* and many species of *Polyporus* had better be fixed in picro-acetic acid and imbedded in celloidin. Young stages of *Cyathus* or *Crucibulum* (bird's-nest fungi) cut easily in paraffin, but the older stages cut much better in celloidin. It is hard to get the very soft, watery forms like *Tremella* into paraffin without shrinking, but sections as thin as 10 μ may be cut in celloidin. While this is too thick to give satisfactory views of such small nuclei, it brings out very clearly the general morphological structures.

THE LICHENS.

The lichens are usually regarded as difficult forms. In younger stages they occasion no trouble, but an old apothecium or a leathery thallus often fails to cut well. Difficulties may be minimized by using prolonged periods. The following schedule has proved satisfactory for the thalli and mature apothecia of *Physcia*, *Usnea*, *Sticta*, *Collema*, *Parmelia*, and *Peltigera:*

1. Chromo-acetic acid (medium solution, p. 28), 2 to 4 days.
2. Wash in water, 6 to 24 hours.
3. Thirty-five, 50, 70, 85, and 95 per cent. alcohols, 6 to 24 hours each.
4. One hundred per cent. alcohol, 2 to 4 days, changing 2 or 3 times.
5. Mixtures of alcohol and xylol, 1 to 2 days.
6. Pure xylol, 6 to 24 hours.
7. Xylol and paraffin on the bath, 1 to 2 days.
8. Paraffin at 54° to 60°, changing once or twice, 3 to 6 days.
9. Imbed in as thin cakes as possible.

Cyanin and erythrosin is a very good stain for lichens. The algæ stain blue and the filaments of the fungus take the red. Where the association of the alga and the fungus is rather loose, as in *Dichonema*, more satisfactory mounts can be made by staining in eosin, or hæm-alum and eosin, and then teasing slightly with needles and mounting in glycerine.

CHAPTER XIII.

BRYOPHYTES.

The Bryophytes, comprising the two groups Liverworts (*Hepaticæ*) and Mosses (*Musci*), present a great diversity of structure, some being so delicate that good preparations are very uncertain, while others are so hard that it is difficult to get satisfactory sections. Between these extremes, however, there are many forms which readily yield beautiful and instructive preparations.

If but one fixing agent should be suggested for the entire group, it would be chromo-acetic acid with ¾ g. chromic acid and ½ cc. acetic acid to 100 cc. of water. It should be allowed to act for at least twenty-four hours, and probably two or three days would be better. Always make an effort to get the material into paraffin, using celloidin only as a last resort for refractory structures which resist infiltration and for very delicate structures which persist in collapsing. As one gains in experience and carefulness, the number of cases which seem to demand celloidin will become fewer and fewer.

Instead of treating forms in a taxonomic sequence, we shall consider first the gametophyte structures under the headings *thallus*, *antheridia*, and *archegonia*, and shall then turn our attention to the *sporophyte*.

HEPATICÆ.

Some of the liverworts are floating aquatics, but most of them grow on logs or rocks or upon damp ground. They are found at their best in damp, shady places. Many of them may be kept indefinitely in the greenhouse. *Riccia*, *Ricciocarpus*, *Marchantia*, *Conocephalus*, *Asterella*, and many others vegetate luxuriously, and often fruit if kept on moist soil in a shady part of the greenhouse, and they do fairly well in the ordinary laboratory if covered with glass and protected from too intense

light. The living plants are very desirable, since they not only furnish the best possible material for habit work and the coarser microscopic study, but they also enable one to secure complete series in the development of the various organs.

The Thallus.— In many cases it will not be necessary to make a special preparation for the study of the thallus, since preparations of antheridia, archegonia, or sporophytes may include good sections of vegetative portions. This is particularly true of forms like *Riccia*, where the various organs are not raised above the thallus. In forms like *Marchantia*, where the antheridia, archegonia, and sporophytes are borne upon stalked receptacles, it is better to make separate preparations to show the structure of the mature thallus. Sections intended to show the structure of the mature thallus should be 15 μ to 25 μ in thickness, but sections to show the growing point and development of the thallus should not be thicker than 10 μ. Material showing apical cells and development of the thallus is easily gotten into paraffin, even in forms like *Ricciocarpus*, which in their mature condition are in danger of collapsing. The apical region of the foliose *Jungermanniaceæ* (*fig. 41*) affords an excellent opportunity for studying the development of the plant body from a single apical cell. If mixtures containing osmic acid are used for fixing, there may be difficulty in the staining, even after using peroxide of hydrogen. Corrosive sublimate-acetic, Carnoy's fluid, or chromo-acetic acid are better for apical regions. A fairly vigorous staining with a mixture of acid fuchsin and iodine green often brings the walls out very sharply. Chromo-acetic acid, followed by Delafield's hæmatoxylin or bismark brown, is good for the apical cells and developing regions, but a light counter-stain with erythrosin improves preparations of the

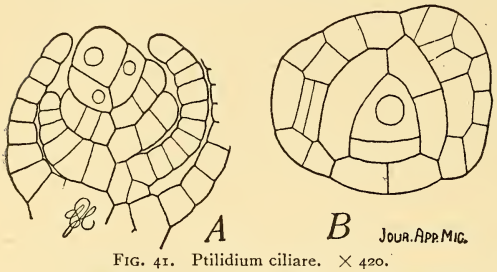

FIG. 41. Ptilidium ciliare. × 420.

A, longitudinal. *B*, transverse section of the apex of the leafy gametophyte. Fixed in Flemming's weaker solution, stained in a mixture of acid fuchsin and iodine green. Ten microns.

mature thallus. After corrosive sublimate-acetic the material may be stained in bulk with alum cochineal or alum carmine, thus giving fairly good preparations and saving considerable labor.

Antheridia.—If you have the material, it is not difficult to get good preparations showing the development of antheridia. In forms like *Asterella*, *Pellia*, etc., cut out a small portion of the thallus bearing the antheridia. The piece should not be more than a quarter of an inch square, and if it can be smaller, so much the better. For early stages of the antheridia of *Marchantia* select young antheridiophores which still lie close to the thallus. These readily cut as thin as 5 μ, and a single slide will usually show a more complete series than is represented in the figure of *Asterella* (*fig. 42*), but after the stalk begins to lengthen, the younger stages become infrequent, and it is not always easy to cut thin sections. Delafield's hæmatoxylin or bismark brown serves very well for such stages as are shown in the figure. The protoplasm of the young antheridia is so dense that the addition of a counter-stain is almost sure to injure the preparation by obscuring the cell walls. For stages older than that represented in *D*, showing the development of the spermatozoid, the paraffin must be rather hard (melting at 55° C. to 65° C.), and the sections should not be thicker than 5 μ, while 2 μ or 3 μ is best. For such stages use the safranin-gentian violet-orange combination, Haidenhain's iron alum-hæmatoxylin with or without a faint trace of erythrosin or orange G, or use a mixture of acid fuchsin and methyl green. Nothing but practice and patience will bring success in such critical work.

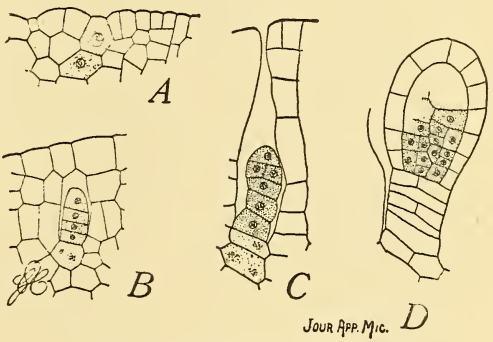

Fig. 42. Asterella hemisphærica. × 255.

Successive stages in the development of antheridia. Fixed in chromo-acetic, stained with Delafield's hæmatoxylin. Section 10 microns thick.

If antherozoids are found escaping, transfer them to a small drop of water on a clean slide, invert the drop over a 1 per cent. solution of osmic acid for two or three minutes, allow the drop to dry up, pass the slide through the flame two or three times, as in mounting bacteria, and then stain sharply in acid fuchsin. This should show the general form of the antherozoid, and will usually bring out the cilia.

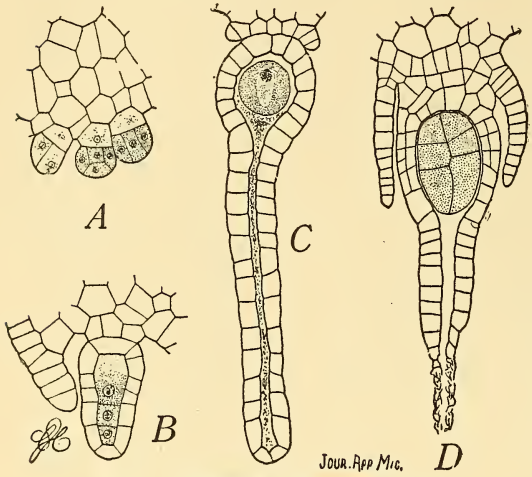

FIG. 43. Marchantia polymorpha. × 400.

A, three early stages in the development of the archegonia. Delafield's hæmatoxylin. *B*, young archegonium showing two-neck canal cells and the central cell before the cutting off of the ventral canal cell. Fuchsin and methyl green. *C*, mature archegonium just ready for fertilization. Safranin-gentian violet-orange. *D*, young embryo. Delafield's hæmatoxylin.

The Archegonia.—The methods for archegonia are practically the same as for antheridia. Too much stress cannot be laid upon the importance of carefully selecting the material. Use very small pieces, and, before placing them in the fixing agent, trim them to such a shape that the position of the archegonia will be accurately known even after the pieces are imbedded in paraffin. For stages like *fig. 43*, *A* and *B*, Delafield's hæmatoxylin is a good stain, and 10 μ is about the right thickness. For stages like *C*, in such forms as *Marchantia*, where the necks are long and often somewhat curved, it is better for general purposes to use sections from 15 μ to 20 μ in thickness. If it is desired to obtain preparations showing the cutting off of the ventral canal cell, the development of the oösphere, and the process of fertilization, the sections should be from 5 μ to 10 μ in thickness, and the same staining may be used as for the development of antherozoids. For archegonia containing young embryos, like that shown in *D*, Delafield's hæmatoxylin without any counter-stain gives beautiful preparations when the staining is well done. It is easier for the beginner

to get good preparations with the safranin-gentian violet-orange combination.

The Sporophyte.—Sporophytes in early stages of development often yield good preparations without very much trouble, but in later stages they are frequently difficult to cut on account of the secondary thickening of the capsule wall and the stubborn extine of the mature spores. It is hard to get *Ricciocarpus* into paraffin without shrinking, and the same thing may be said of other forms which have such loose tissue with large air cavities. For stages like that shown in *fig. 44*, as well as for older sporophytes, it will be found more uniformly satisfactory to use celloidin, and cut the sections from 20 μ to 30 μ thick.

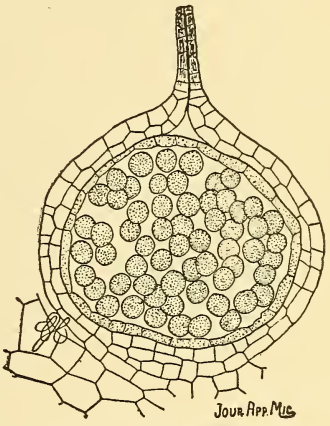

Fig. 44. Ricciocarpus natans. × 104.

Young sporophyte inclosed in the archegonium. Spore mother-cell stage. All the cells of the sporophyte except a single peripheral layer (dotted in the figure) produce spores. Fixed in picro-acetic acid and stained in Delafield's hæmatoxylin. Celloidin section 30 microns in thickness.

For a study of archegonia, antheridia, young sporophytes, and also for the development of the thallus, it is better, even in forms like *Ricciocarpus*, to use paraffin. Prolonged fixing in chromo-acetic acid (two to six days), thorough dehydrating, and gradual transfer from alcohol to xylol, and from xylol to paraffin, and also a moderate temperature in the bath (not more than 50° C.), will often bring the material through in fine condition.

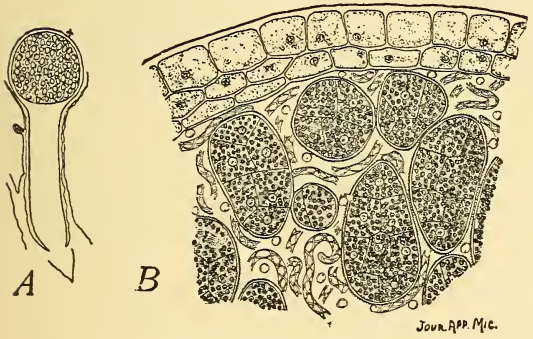

Fig. 45. Pellia epiphylla.

A, habit sketch of sporophyte. × 10. *B*, small portion of sporophyte (at × of *A*), showing the capsule wall, the spores, and the elaters. Fixed in chromo-acetic acid and stained in cyanin and erythrosin. Ten microns.

Forms like *Pellia* cut well in paraffin, especially in younger stages, but even in case of mature sporophytes it is not necessary to resort to celloidin. In *Pellia* and *Conocephalus* the spores are very large and have a rather thin wall. Both these genera show a peculiar, intrasporal development of the gametophyte, *i. e.*, the gametophyte develops to a considerable extent before it ruptures the spore wall. (*Fig. 45.*) For the older sporophytes of *Marchantia* it is better not to cut the whole receptacle, but

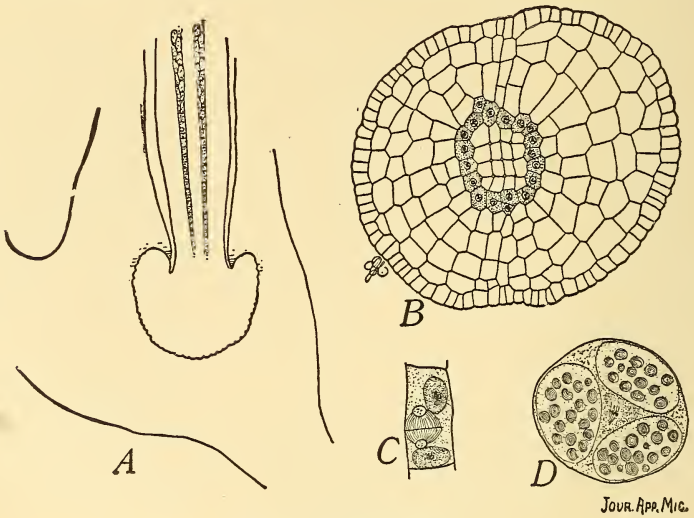

FIG. 46. Anthoceros laevis.

A, longitudinal section of lower portion of sporophyte imbedded in the gametophyte. × 45. *B*, transverse section of lower portion of sporophyte. × 200. Delafield's hæmatoxylin. Ten microns. *C*, vegetative cell from lower portion of the sporophyte. × 560. Fixed in Flemming's weaker solution and stained in a mixture of acid fuchsin and iodine green. Five microns. *D*, spore mother-cell showing three of the four chloroplasts with numerous starch grains. The nucleus is in the metaphase of the first division. × 560. Fixed in Flemming's weaker solution, stained in safranin-gentian violet-orange. Five microns.

rather to remove the branches so that they may be cut separately. For the very best preparations of mature sporophytes it will pay to trim away the gametophyte structures, leaving only enough to show the foot with a few of the surrounding cells. Sections 5 μ to 10 μ thick can be made without much difficulty from material prepared in this way.

Among the Bryophytes no form affords a better opportunity for studying the development of spores than *Anthoceros*,

since a single longitudinal section of the sporophyte may show all stages, from earliest archesporium to mature spores (*fig. 46*). For studies like *A* and *B*, chromo-acetic material cut 10 μ thick and stained in Delafield's hæmatoxylin is very good. The starch grains in the chloroplasts take a beautiful violet color with the safranin-gentian violet-orange combination. It is very difficult, however, to bring out the details of nucleus or chloroplast on account of the minute size of these structures. The drawings from which *C* and *D* were reproduced were made with a one-sixteenth oil immersion objective. The drawings, like all the others illustrating the Bryophytes, were reduced one-half by photography.

CHAPTER XIV.

BRYOPHYTES.

MUSCI.

Material for a study of the mosses is much more abundant, and a series of stages in the development of the various organs is easily secured; but it is much more difficult to obtain good preparations, because so many of the structures are hard to cut. Chromo-acetic acid is to be recommended as the most satisfactory fixing agent, but where structures are refractory and very likely to make trouble in cutting, it will often be found more satisfactory to use picro-acetic acid in the 70 per cent. alcohol, since material fixed in this reagent does not become as hard or as brittle as that fixed in any of the chromic-acid series.

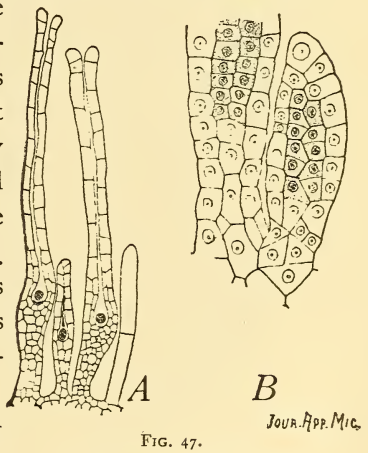

FIG. 47.

A, archegonia of Webbera candicans. × 104. Celloidin section. Twenty microns. *B*, young antheridia of Polytrichum commune. × 420.

Antheridia.— It is easy to find material for a study of antheridia, because, in so many cases, the antheridial plants can be detected at once without even a pocket lens. *Funaria*, with its bunch of antheridia as large as a pin-head, is extremely common everywhere. Spring is the best time to collect it, but it is found fruiting in the autumn and sometimes in summer; besides, it is easily kept in the greenhouse, where it may fruit at any time. *Bryum proliferum* has a still larger cluster of antheridia, which may be seen at a distance of several yards. *Polytrichum* also has a large cluster of antheridia surrounded by reddish leaves, so that the whole is sometimes called the moss "flower." In making preparations of *Polytrichum* these colored leaves should be carefully removed

after the material has been gotten into 70 per cent. alcohol. A single antheridial plant of *Polytrichum* often furnishes a fairly complete series of stages in the development of antheridia. (*Fig. 47.*) In all cases the stem should be cut off close up to the antheridia, for many of the moss stems cut like wire. It is not necessary to use celloidin for antheridia, nor is it desirable, except where sections from 20 μ to 50 μ thick are wanted for habit work. Delafield's hæmatoxylin is recommended for staining.

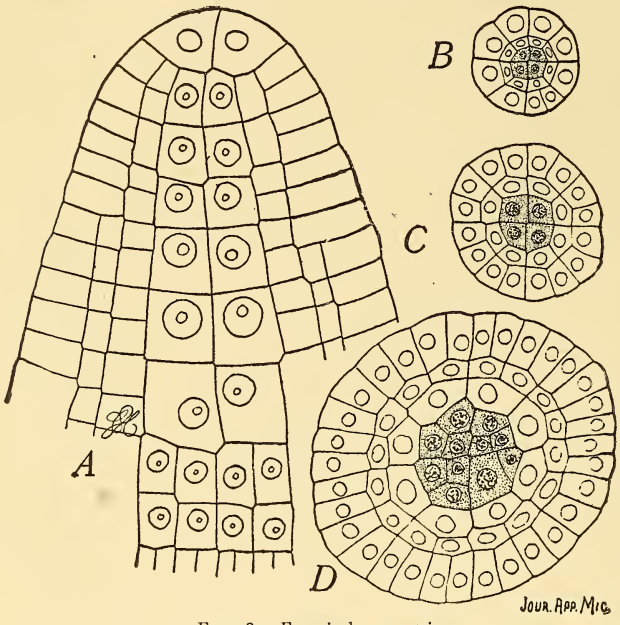

Fig. 48. Funaria hygrometrica.

A, apex of young sporophyte showing endothecium and amphithecium. × 420. Chromo-acetic acid and Delafield's hæmatoxylin. Ten microns. *B*, *C*, and *D*, transverse sections of a sporophyte of the same age as *A*, taken at three different levels. × 255. Ten microns.

Archegonia.— Since the necks of the archegonia are usually long and more or less curved, it is necessary, for habit work, to cut sections as thick as 20 μ or 30 μ in order to get a view of an archegonium in a single section. Celloidin may be used for such preparations, but for the development of the archegonium, the oösphere, the canal cells, and also for the process of fertilization, it is better to use paraffin. For the thick celloidin sections the material may be stained in bulk in alum cochineal, but thin paraffin sections should be stained on the slide with more critical stains. (*Fig. 47.*)

The Sporophyte.— It is often difficult to get good mounts of sporophytes. In the younger stages the calyptras are likely to interfere with cutting, while in the older stages the peristome, or

hard wall of the capsule, occasions the trouble. If an attempt is made to remove the calyptra in young stages, like *A* of *fig. 48*, the apex of the sporophyte usually comes with it. While picro-acetic acid material cuts more easily, chromo-acetic acid followed by Delafield's hæmatoxylin gives so much sharper differentiation in stages like those shown in *fig. 48* that it is better

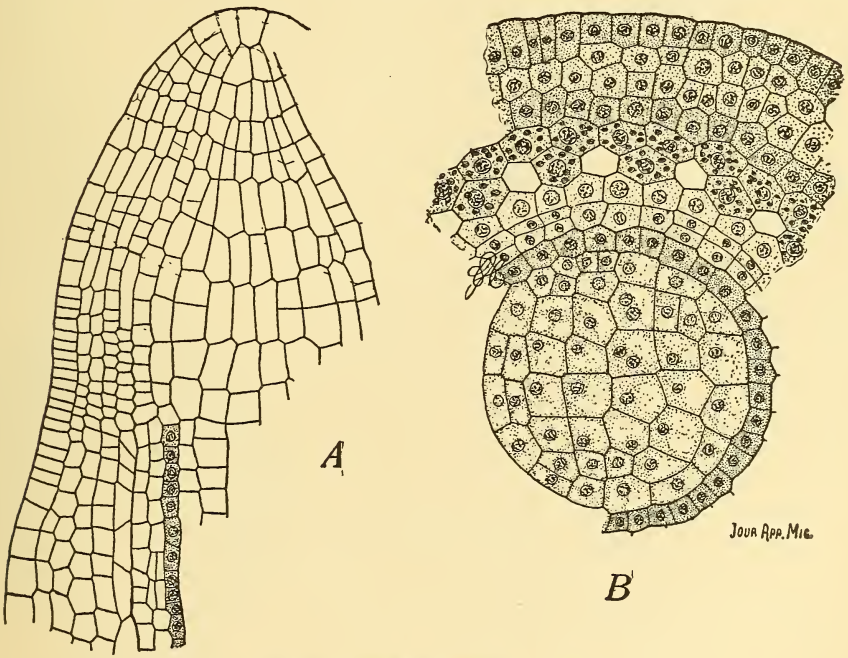

Fig. 49. Funaria hygrometrica. × 420.

A, longitudinal section of capsule. *B*, transverse section of capsule of about the same age as *A*. The columella, archesporium, outer spore case, two layers of chlorophyll-bearing cells, and the beginning of the air spaces can be distinguished at this stage. Delafield's hæmatoxylin and erythrosin. Ten microns.

to use harder paraffin (55° to 60° C.) and make an effort to get preparations from chromic material.

Stages like that shown in *fig. 49* are cut with comparative ease, for the calyptra is easily removed, and the capsule wall is not yet hard enough to occasion any difficulty. The cell walls are so easily stained in moss capsules that a light counter-stain with erythrosin or acid fuchsin may be used to bring out the

L. of C.

cytoplasm and plastids without appreciably obscuring the cell walls. *Funaria* and *Bryum* afford an excellent study in the development of the capsule, since all the structures of a highly differentiated moss sporophyte are present, and *Bryum* is particularly easy to cut in stages like those shown in *fig. 50*.

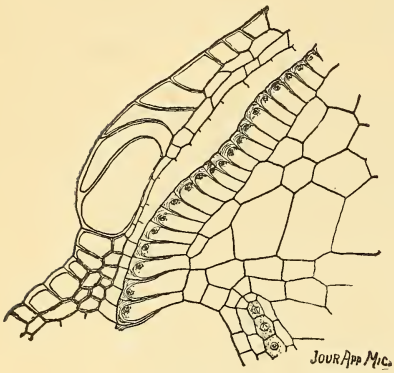

FIG. 50. Bryum. × 200.

Portion of a nearly mature capsule showing operculum, annulus, peristome, and three cells of the sporogenous tissue. Fixed in Flemming's weaker solution, stained in safranin and Delafield's hæmatoxylin. Fifteen microns.

Sporophytes, in their more mature stages, are almost sure to present considerable difficulty in cutting. For general work fairly good preparations may be gotten from celloidin material, but it is worth while to try paraffin, for it is sometimes successful, and when it does succeed it is far superior. As soon as the cell walls begin to thicken, as in the development of the peristome, safranin is an excellent stain, and this, followed by Delafield's hæmatoxylin, will give an elegant differentiation in the older stages of the sporophyte. After capsules have begun to turn brown it will be almost impossible to infiltrate them unless they are pricked with a needle.

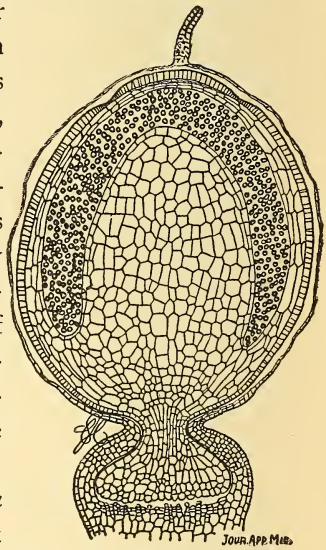

FIG. 51. Sphagnum. × 24.

Longitudinal section of mature sporophyte, showing also the upper portion of the pseudopodium and the calyptra. Chromo-acetic acid, Delafield's hæmatoxylin. Paraffin. Ten microns.

The mature sporophytes of *Sphagnum* (*fig. 51*) are exceptionally hard to cut. It will be worth while to prick the capsule with a needle when the material is collected. This will allow the fixing agent to penetrate readily, and will also facilitate the infiltration of paraffin or celloidin. The puncture causes only a slight damage, and need not reach the really valuable portion which is to furnish the median longitudinal sections.

Protonema and teased mounts of antheridia and archegonia may be made directly in 50 per cent. glycerine without fixing or staining. While this method is often recommended, we have found it better to use 10 per cent. glycerine and allow it to concentrate. Mounts made in this way retain their green color for a long time.

CHAPTER XV.

PTERIDOPHYTES.

This group, including the Filicineæ, Equisetineæ, and Lycopodineæ, or, more popularly, the ferns, horsetail rushes, and club mosses, is familiar to everyone. Material is abundant, and so easily recognized that anyone who pays a little attention to collecting can, in a single season, get a fine supply for a study of the group. Some desirable forms may not be present in all localities, but these will be few and can be obtained at a reasonable price from those who make a business of collecting.

FILICINEÆ.

Without attempting to follow any taxonomic sequence, the methods of preparing the various structures of the homosporous forms will be presented, and then the peculiarities of the heterosporous members will be considered.

The Prothallia.—Ripe spores of some fern or other can be obtained at any greenhouse at any time in the year, and spores of most of our native ferns germinate well and produce good prothallia, even if the sowing is not made for several months after the spores have been gathered.

Fine prothallia of *Pteris aquilina* have been grown two years after the spores were gathered. Some, however, must be sown at once, or they will not germinate at all. The spores of the common *Osmunda regalis*, and probably of the other members of the genus, must be sown as soon as ripe, or they fail to germinate. The prothallia of *Osmunda regalis*, if carefully covered with glass, may be kept for a long time. Prothallia of this fern in the writer's laboratory produced ribbon-like outgrowths three-sixteenths of an inch wide, and often more than two inches in length. These prothallia continued to produce archegonia, antheridia, and the ribbon-like outgrowths for more than a year,

when they suddenly "damped off." *Pteris aquilina* and many other ferns often furnish a good supply of antheridia in three weeks after sowing, and the archegonia appear soon after, but it is well to make sowings six weeks before material is needed for use. In *Pteris aquilina* and in many others, if the spores are sown too thickly, only antheridial plants will be obtained. If prothallia are to produce archegonia, they must have sufficient room and nutrition. If there are no greenhouse facilities and the prothallia must be grown in the laboratory, it is a good plan to take a glass dish, ten or twelve inches in diameter and about two inches deep, put a layer of broken pieces of flower pots on the bottom, cover this with a layer of rich loam, and over this sprinkle a layer of fine, clean sand, since sand is much more easily washed away from the rhizoids than is the loam. The whole should now be thoroughly wet, but not so as to have water standing on the bottom. Sow the spores and cover with a tightly fitting pane of ground glass. There should be no need for moistening the culture again, for prothallia can be kept fresh and vigorous for several months, or even for a year, without any wetting. When it is desired to secure fertilized material, sprinkle the prothallia with water, and the young sporophytes will soon appear. If greenhouse facilities are available, any gardener can grow prothallia in abundance without any directions from those who want the material.

The peculiar tuberous prothallia of *Botrychium* are seldom found except by the experienced collector. The older prothallia, however, may be found by anyone who is able to recognize *Botrychium* when he sees it. Dig up young plants not more than three or four inches in height, and the prothallia, which persist for years, will often be found still attached. They are easy to cut and may be handled like other prothallia.

Fern prothallia of the usual type are excellent objects for testing fixing agents, since the prothallia, while still in the fixing agent, may be examined with the microscope, and fluids which cause plasmolysis may be rejected. It will sometimes happen that plasmolysis may be avoided by varying the proportions of

the ingredients of a fixing agent. Chromo-acetic acid with about 0.6 g. chromic acid and 0.4 cc. of acetic acid to 100 cc. of water will seldom cause plasmolysis, and will usually insure good fixing. It is a mistake to suppose that because prothallia are such delicate objects the fixing will take but a few minutes. We should recommend at least twenty-four hours in chromo-acetic or Flemming's fluid and two or three days will do no harm and may be better. If hot corrosive sublimate-acetic acid or hot picro-acetic be used, the fixing requires only two or three minutes, but results are not as uniformly successful as with members of the chromic-acid series. After any of the chromic-acid series, two or three hours' washing in water will be sufficient, if the water be changed as often as it becomes in the least degree discolored.

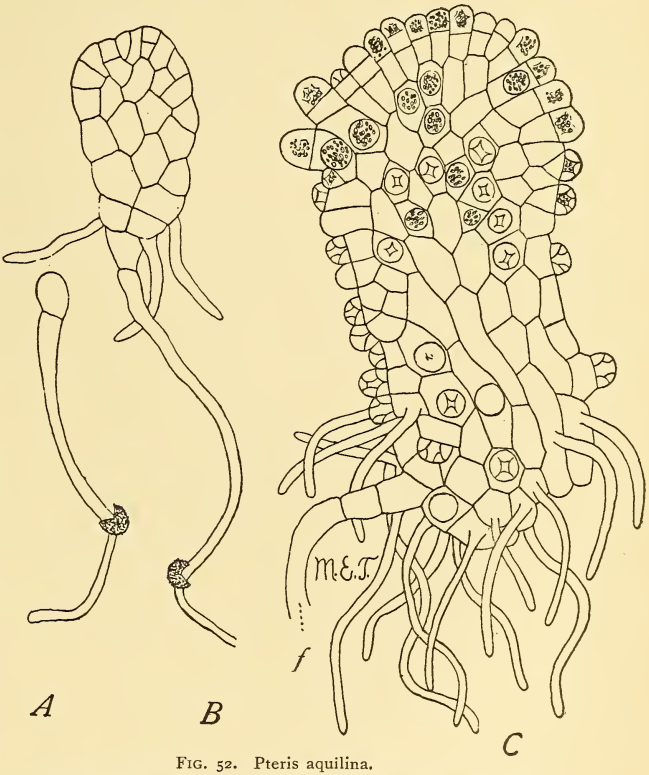

FIG. 52. Pteris aquilina.

A, filamentous stage. *B*, the apical cell has been established and several segments have been cut off. The figure shows the initial rhizoid, and also three rhizoids coming from the main body of the prothallium. *C*, an older prothallium covered with antheridia in various stages of development. From a glycerine mount, fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin. (MISS M. E. TARRANT.)

If preparations are to be mounted whole, as shown in *fig. 52*, they should be stained as soon as the washing is finished. Any of the following methods gives good results:

a. Stain in Mayer's hæm-alum six hours or over night,

wash in water one or two hours, and transfer to 10 per cent. glycerine.

b. Stain in Delafield's hæmatoxylin thirty minutes, wash in water one or two hours, decolorize in water acidulated with hydrochloric acid (3 drops of HCl to 100 cc. water) one to

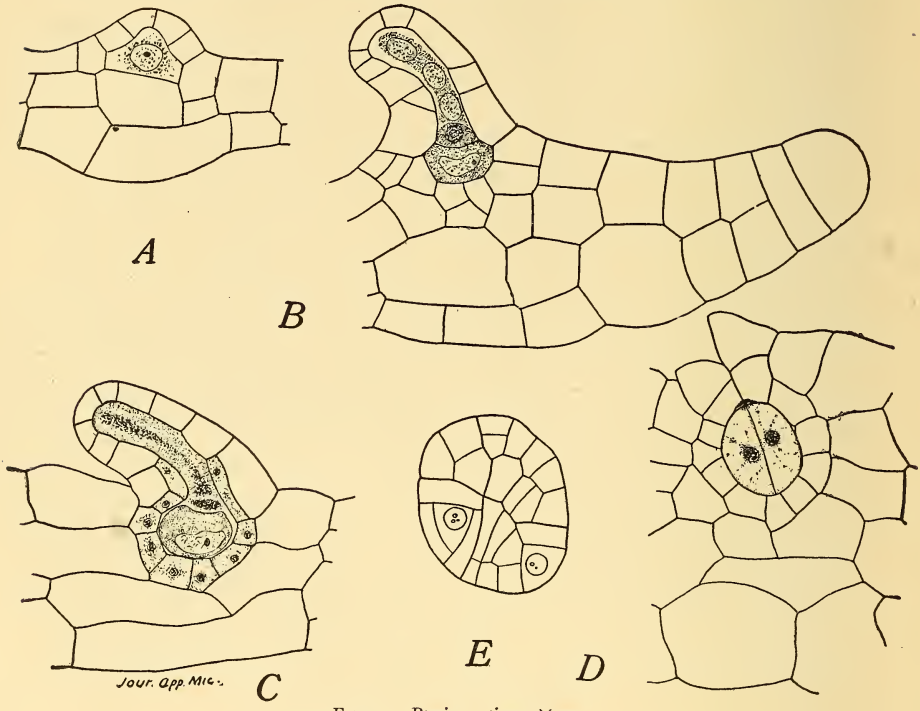

FIG. 53. Pteris cretica. × 250.

A, early stage in the development of the archegonium. *B*, later stage showing the oösphere, ventral canal cell, and *three* nuclei in the neck canal. *C*, still later stage almost ready for fertilization. The ventral and neck canal cells are breaking down, and the oösphere is nearly mature. Cells surrounding the oösphere have become richer in protoplasmic contents, and stain more deeply. *D*, first division of the embryo. *E*, young embryo still showing the outlines of the four quadrants. The apical cell in the lower left quadrant has cut off the first layer of the root cap. All drawn from material stained in bulk in alum carmine, a method not to be recommended.

thirty minutes—the time can be determined only by experiment—wash in water until the rich purple color of the hæmatoxylin replaces the red due to the acid, and then place in 10 per cent. glycerine.

c. Stain with Delafield's hæmatoxylin as in *b*, and after the last washing in water stain two to four minutes with an aqueous

solution of eosin, wash thoroughly in water, and transfer to the 10 per cent. glycerine. Instead of using the acid alcohol after the hæmatoxylin, the eosin may be allowed to act for several hours, and then 1 per cent. acetic acid may be used for a few minutes. Wash very thoroughly in water, and, if the stains appear satisfactory, transfer to 10 per cent. glycerine as before.

d. Use a 2 per cent. solution of iron alum two hours, wash in water five minutes, stain in ½ per cent. hæmatoxylin two to six hours, wash in water five minutes, and then treat again with iron alum until the stain is satisfactory. Wash thoroughly in water and transfer to 10 per cent. glycerine, which, as usual, will concentrate sufficiently for mounting in three or four days.

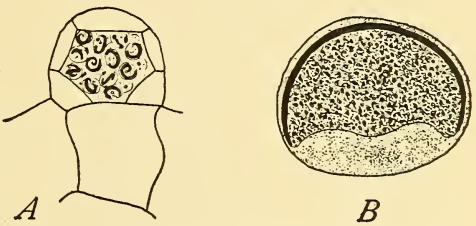

FIG. 54. Pteris cretica.

A, section of a nearly mature antheridium showing the antherozoids inside. × 333. *B*, a developing antherozoid showing the blepharoplast drawn out into a deeply staining band. × 1900. Fixed in chromo-acetic acid and stained in Haidenhain's iron alum-hæmatoxylin.

For paraffin sections such as are shown in *figs. 53* and *54*, the material should be passed through the alcohols, allowing about three or four hours for each grade. The mixtures of xylol and absolute alcohol should take about six hours, and as soon as the pure xylol has been added, the piece of paraffin may be added at the same time. In the bath two or three hours will give good results. It would be worth while to determine the duration of the bath for such objects. Some workers claim that ten or fifteen minutes is amply sufficient, and that there is less danger from shrinking, while others think that several hours is better, and that two or three days will do no damage, if the fixing has been thorough and the temperature is not allowed to become higher than 50° C. If bergamot oil be used instead of xylol, there is less danger of collapse previous to the bath, but the bath itself must usually be more prolonged, since the bergamot oil is not so easily gotten rid of as is the xylol.

For morphological purposes sections 15 μ to 20 μ thick are better than thinner ones. Delafield's hæmatoxylin, with or without

a counter-stain with erythrosin, is good for such sections as are represented in *fig. 53*. If very thin sections are wanted for cytological study, it is better to use the safranin-gentian violet-orange combination. For such views of antheridia as are shown in *fig. 54*, Haidenhain's iron alum-hæmatoxylin seems to bring out the blepharoplast (centrosome) most sharply.

The Sporophyte.—Methods for young sporophytes like those shown in *fig. 53*, *D* and *E*, are the same as for archegonia and antheridia.

Root tips to show the prominent apical cell are easily imbedded in paraffin. They should be cut 15 μ to 20 μ thick. Delafield's hæmatoxylin, without any contrast stain, is best for bringing out the prominent apical cell and its segments.

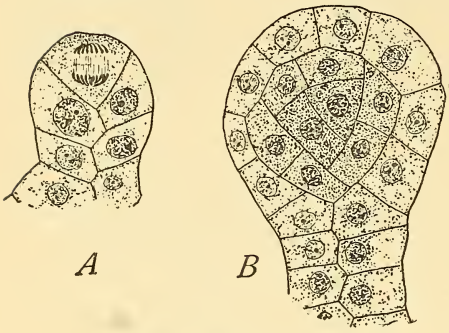

FIG. 55. Pteris cretica. × 560.

A, young stage in the development of the sporangium. *B*, older stage showing the tapetum. Fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin and erythrosin.

Sporangia should also be cut in paraffin. *Pteris* is a good form for sporangia, since the long marginal sorus makes it possible to get an immense number of median longitudinal sections of sporangia in a single preparation. (*Fig. 55.*)

Pteris cretica can always be found in fruit in greenhouses. Select a series of stages. The leaf should be cut with a razor—not with scissors—into pieces about one-fourth of an inch long. If sections of the whole leaf are not wanted, only the marginal sorus need be cut off; in this way a much greater number of sporangia may be gotten upon a slide. *Aspidium* and *Cyrtomium* give beautiful views of the indusium covering the cluster of sporangia. The most satisfactory preparations will be gotten from material in which the sporangia have not yet begun to turn brown.

Botrychium furnishes excellent and usually accessible material for studying the development of sporangia of the eusporangiate type. For the archesporium and early stages in the development

of the sporangia the material should be collected in the latter part of August or in September. When the sporangia for one year are ready to shed their spores, the sporangia for the next year will be found in early stages of development. When a frond is found, dig the plant up very carefully and remove the large frond. At its base will be found the frond for the next season, the sterile portion bent over so that its tip is directed downward and the fertile portion — one-fourth to three-fourths of an inch in length — projecting from the ventral surface of the sterile frond and directed upward. It is worth while to preserve the whole bud and also portions of the stipe, rhizome, and roots. These vegetative parts should be cut, with a razor or very sharp knife, into pieces about one-fourth of an inch in length. The larger roots are better for transverse sections. The root tips are unusually favorable for preparations of the apical cell. The upper part of the rhizome cuts easily, but all parts of this plant, even the older portions of the rhizome, can be cut in paraffin. Delafield's hæmatoxylin or iron alum-hæmatoxylin gives fine preparations of the young sporangia. The older sporangia, from the mother-cell stage up to the shedding of the spores, stain better in cyanin and erythrosin or in the safranin-gentian violet-orange combination. The following schedule for paraffin sections will give elegant mounts of the root, stipe, and rhizome:

1. Stain in safranin, about 24 hours.
2. Fifty per cent. alcohol until little or no safranin is left in the cellulose walls. Use a very weak acid alcohol, if necessary.
3. Delafield's hæmatoxylin, 5 to 10 minutes.
4. Water, 15 minutes.
5. Thirty-five and 50 per cent. alcohol, 10 seconds each.
6. Acid alcohol — the same as was used for the safranin — a few seconds.
7. Seventy per cent. alcohol until the purple color returns. The lignified walls should now show a brilliant red and the cellulose walls a rich purple. If either stain is too deep or too faint, do not proceed any farther before making the necessary correction. If the safranin washes out, and the hæmatoxylin is too intense, do not use any acid alcohol in the second step and shorten the period in the hæmatoxylin.
8. Eighty-five and 95 per cent. alcohol, a few seconds each.
9. Hundred per cent. alcohol, 30 seconds to 1 minute.
10. Xylol until cleared.
11. Balsam.

The spore mother-cells of *Osmunda* are excellent for a study of mitosis. The young sporangia of *O. cinnamomea* and *O. Claytoniana* show the mother-cell stage in the autumn, but the division into spores does not occur until the following spring, in the vicinity of Chicago the mitotic figures being found during the latter part of April. *O. regalis* does not reach the mother-cell stage in the autumn. Material for mitosis should be collected during the first two weeks in May. The material may be fixed in the medium chromo-acetic solution or in Flemming's weaker solution. Sections should not be thicker than 10 μ, and 5 μ will be found more satisfactory.

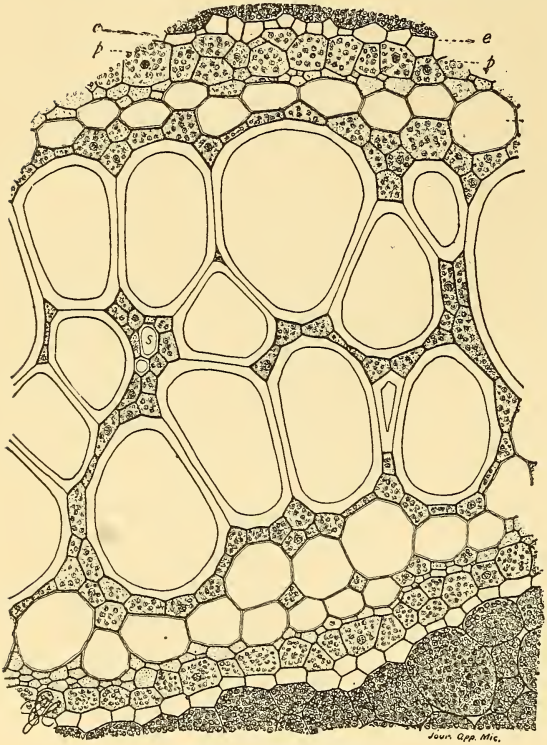

FIG. 56. Pteris aquilina.

A part of a transverse section of the vascular bundle of the rhizome. × 166. *e*, endodermis. *p*, pericycle. *st*, sieve tube. *t*, scalariform tracheid. Drawn from a celloidin section from material fixed in picro-acetic acid and stained in safranin and Delafield's hæmatoxylin.

Preparations of the woody structure of the sporophyte are easily made. The rhizome of *Pteris aquilina* affords as good material as any (*fig. 56*). In digging up rhizomes, do not merely dig down until the rhizome can be grasped and then pull it up, for such material is sure to show the pericycle of the bundles torn away from the parenchyma. Dig carefully around the rhizome and then cut off with a very sharp knife pieces about two inches in length. Put the fresh rhizome into the hand microtome and cut as thin sections as possible. Keep the knife wet with 95 per cent. alcohol and put the sections into 95 per cent.

alcohol as fast as they are cut. Fifteen or twenty minutes is sufficient for fixing. Pass down through the grades of alcohol, about two or three minutes in each grade. Stain in safranin twenty-four hours, wash in water ten minutes, and then stain in Delafield's hæmatoxylin ten to twenty minutes. Pass through the alcohols, about one minute in each grade, at 70 per cent. alcohol transferring to acid alcohol from one to five seconds; then pass on, clear in xylol, and mount in balsam. If the stain is successful, the xylem should show a brilliant red, and the cellulose walls a rich purple.

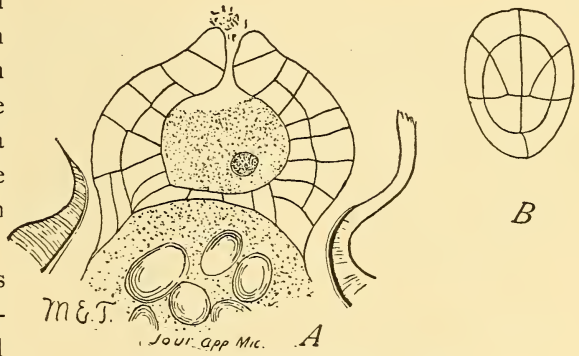

FIG. 57. Marsilea quadrifolia. × 333.

A, apex of megaspore with archegonium containing the oösphere. Large starch grains are shown beneath the archegonium. *B*, young embryo. Both fixed in picro-acetic acid and stained in Delafield's hæmatoxylin. (MISS M. E. TARRANT.)

Another method is to stain for about twenty minutes, or several hours, in iodine green or methyl green, rinse in 70 per cent. alcohol until the green is prominent only in the xylem, then stain for from one to three minutes in acid fuchsin (1 per cent. solution in 70 per cent. alcohol), dehydrate rapidly, clear in xylol, and mount in balsam. The xylem should have a sharp bright green color, and the cellulose a bright red.

The most beautiful preparations may be obtained by imbedding in celloidin and staining in safranin and Delafield's hæmatoxylin. These stains allow a use of acid which extracts all color from the celloidin and still leaves a sufficient amount in the tissues. The large apical cells of rhizomes are easily cut in either paraffin or celloidin.

The megaspores and microspores of *Marsilea* are easily obtained (*figs.* 57 and 58). Cut away a portion of the hard sporocarp and place the sporocarp in a dish of water. The gelatinous ring with its sori will sometimes come out in a few

minutes. In less than twenty-four hours the microspores, starting from the one-cell stage, will produce the mature antherozoids. The development of the megaspore is equally rapid. Embryos are abundant in two or three days. For morphological work, picro-acetic acid, used hot, is very good, since the material does not occasion so much difficulty in cutting. Chromo-acetic material allows better staining, but the cutting is more uncertain. It is best to prick the megaspores with a needle while they are in the fixing fluid, in order to facilitate the infiltration of paraffin. Better mounts of the microspores can be obtained if the troublesome megaspores be picked out from the sorus while the material is still in the fixing agent or the alcohols. The megaspores must be imbedded in rather hard paraffin, and one must expect to hone the knife thoroughly before it can be used again, for when the knife strikes a megaspore of one of the heterosporous pteridophytes, it seems like striking a grain of sand.

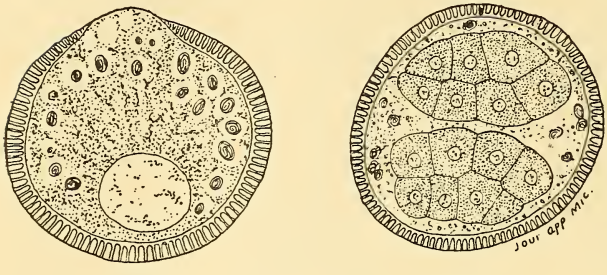

FIG. 58. Marsilea quadrifolia. × 560.

A, microspore before germination. *B*, microspore with antheridia nearly mature. Fixed in chromo-acetic acid and stained in safranin-gentian violet-orange.

The following schedule will usually give good sections of the hard megaspores of the heterosporous pteridophytes, whether they are to be cut separately or in their sori or strobili. The essential features of the method are suggested by Miss F. M. Lyon's work on *Selaginella:*

1. Chromo-acetic acid, medium solution, 2 to 6 days.
2. Wash in water, 1 day.
3. Thirty-five to 95 per cent. alcohol, 1 day each.
4. One hundred per cent. alcohol, 3 or 4 days, changing several times.
5. Mixtures of alcohol and xylol, 2 days.
6. Pure xylol at 53° C., 1 or 2 days.
7. Add paraffin to the xylol and keep at 53° C. for 2 or 3 days.

8. Pure paraffin 53° C., 3 or 4 days, and then in harder paraffin at 60° C. to 70° C., 2 or 3 days.
9. Imbed in rather thin cakes.

While the method is tedious, it is worth the trouble, for even old strobili of *Selaginella* yield smooth ribbons at 5 μ.

As *fig. 57* suggests, the mature archegonia, and especially the young embryos, may be removed from the top of the megaspore and cut with perfect ease.

The spermatozoid, which in *Marsilea* has an unusually large number of turns in the spiral, may be mounted by methods already described.

CHAPTER XVI.

PTERIDOPHYTES.

EQUISITINEÆ.

The prothallia of *Equisetum* are easily grown by the method already described for the Filicineæ, but the spores must be sown as soon as ripe, because they fail to germinate if kept more than a few days. In the vicinity of Chicago the spores of *Equisetum arvense* are shed during the latter part of April. The methods for preparing the prothallia are the same as for the Filicineæ. The sporangia are harder to cut, but good preparations should be secured from paraffin material. *E. arvense* is abundant everywhere, and is to be preferred on account of the comparative ease with which the sporangia and other portions of the fertile shoot can be cut. Longitudinal sections of the younger strobili show various stages in the development of the spores, the more advanced stages being found at the base of the strobilus. Tetrads may be found at the base of the strobilus, while the spore mother-cells at the apex are still undivided. Of course, it is impossible to stain a longitudinal section of such a strobilus so that all stages will be satisfactory. For the beginner, at least, this is not a serious objection, for he will be almost sure to secure some stage beautifully stained. The experienced worker, who is able to control his staining with more precision, will prefer transverse sections. Material for sporangia should be obtained as soon as the fertile shoot appears above ground, and if it can be obtained earlier, so much the better. When the spores are shed, the young sporangia which are to develop the next year can already be detected. The safranin-gentian violet-orange combination can be recommended for the development of the mother-cell and the formation of tetrads, but Delafield's hæmatoxylin or Haidenhain's iron alum-hæmatoxylin will be more satisfactory for earlier stages.

The roots are very small, but have large cells and easily yield good preparations. In case of such small objects it is a good plan to add a few drops of eosin to the alcohol during the process of dehydrating, in order that the material may be seen more easily. The slight staining does no damage, even if more critical stains are to be used after the sections are cut. It is easy to get longitudinal sections of the roots by cutting transverse sections of the nodes. In *E. arvense* these roots at the nodes are quite numerous. Sections of the stem of the fertile shoot of *E. arvense* are easily cut in paraffin or celloidin, but sections of the stem of *E. hiemale* or similar species do not cut in paraffin, and results are rather uncertain even in celloidin. The growing points of stems, however, may be cut with ease in paraffin. *E. arvense* is particularly favorable on account of the numerous apical cells which may be found in a single preparation. Delafield's hæmatoxylin, used alone, is good for the apical cells, but for sections of older stems a slight counter-stain with erythrosin will improve the mount.

CHAPTER XVII.

PTERIDOPHYTES.

LYCOPODINEÆ.

It is not very difficult to get paraffin sections of young sporangia of *Selaginella*, but the method just recommended for *Marsilea* should be resorted to for the older strobili (*fig. 59*).

The older megaspores had better be pricked with a needle and cut one at a time. A slight puncture at the basal portion of the megaspore does no damage and insures a thorough infiltration. If the megaspores are imbedded separately, they will usually orient themselves so that sections perpendicular to the paraffin cake will show the most instructive views of the gametophyte structures.

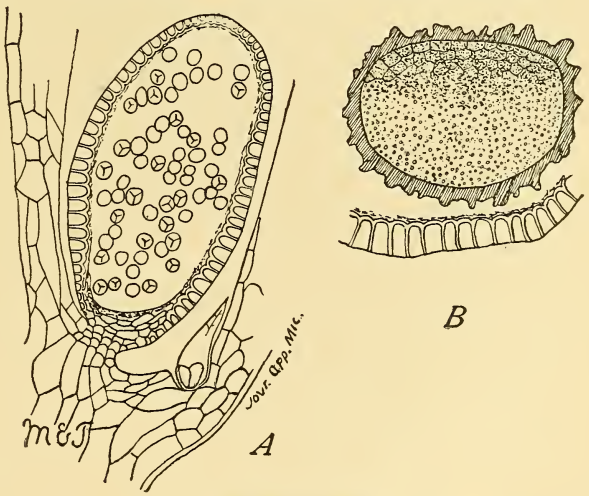

FIG. 59. Selaginella Mertensii. × 93.

A, microsporangium containing microspores. *B*, a megaspore showing the beginning of the prothallium. Fixed in picro-acetic acid and stained in Delafield's hæmatoxylin. (MISS M. E. TARRANT.)

Delafield's hæmatoxylin, used alone, is a good stain. The safranin-gentian violet-orange combination gives a brilliant differentiation in stages like *fig. 59, B*. The microspores of *Isoetes* offer the same difficulties. Cyanin and erythrosin is a fine combination for the reserve food-stuffs in *Isoetes* macrospores.

For young sporangia of *Isoetes*, the leaves should be cut off about one-eighth of an inch above the sporangia, and the stem

should be cut off below, leaving just enough to hold the sporangia together. Cut longitudinal sections and stain in Delafield's hæmatoxylin, with or without the addition of a light touch of erythrosin. The older sporangia had better be removed and cut separately. Transverse sections of the stem are very interesting. Young stems and the apices of old ones cut well in paraffin, but older stems will give more satisfactory results in celloidin.

It is much easier to get good preparations of the sporangia of *Lycopodium*, since there are no megaspores with their hard walls. Delafield's hæmatoxylin, without any contrast stain, will bring out the developing sporangia, which are usually to be found even after the sporangia in the lower part of the strobilus are beginning to shed their spores. The directions for the rhizome of the Filicineæ will also serve for the stem of *Lycopodium*.

CHAPTER XVIII.

SPERMATOPHYTES.

In this immense group we cannot hope to give even approximately complete directions for making preparations, but must be content to give a few hints which may prove helpful in collecting material and in securing mounts of the more important structures of the flowering plants. We shall consider the gymnosperms and the angiosperms separately, although in many respects the technique is the same for both.

GYMNOSPERMS.

Since *Pinus* is a characteristic type, we shall describe methods for demonstrating various phases in the life-history of this genus, hoping that the directions will enable the student to experiment intelligently with other forms.

Spermatogenesis.— In October the clusters of staminate cones which are to shed their pollen in the coming spring are already quite conspicuous. The cones should be picked off separately, and the scales should be carefully removed so as to expose the delicate greenish cone within. At this time the archesporial cells are easily distinguished. Material collected in January, or at any time before growth is resumed in the spring, shows about the same stage of development. If it is desired to secure a series of stages with the least possible delay, a branch bearing numerous clusters of cones may be brought into the laboratory and placed in a jar of water. Growth is more satisfactory in case of branches broken off in the winter than in those brought in before there has been any period of rest. The material can be examined from time to time, and a complete series is easily secured. The karyokinetic figures in the pollen mother-cells furnish exceptionally instructive preparations. Staminate cones which will yield karyokinetic figures can be selected with considerable certainty by examining the fresh material. Crush a

microsporangium from the top of the cone and one from the bottom, add a small drop of water and a cover to each, and examine. If there are pollen tetrads at the bottom, but only undivided spore mother-cells at the top, it is very probable that longitudinal sections of the cone will yield the figures. If a drop of methyl green be allowed to run under the cover, it will enable one to see whether figures are present or not. When desirable cones are found, they should be cut longitudinally into two halves. The later stages, showing the germination of the microspores, furnish better sections if the cones are cut transversely into small pieces about three-sixteenths of an inch thick. It is very easy to get excellent mounts of the pollen just at the time of shedding. Shake a large number of cones over a piece of paper, thus securing an abundance of material. Fix in chromo-acetic acid, wash in water (a few minutes is sufficient, and the water need not be changed), pass through the alcohols, allowing each to act for about two hours, make the usual gradual transition from alcohol to xylol, and from xylol to paraffin. It is best that the material should be in a small bottle not more than one-fourth of an inch in diameter; at any rate, the pollen should be in such a bottle during infiltration, which should not require more than two or three hours, although a longer period does no harm if the temperature does not rise above 52° or 53° C. Now put the lower portion of the bottle into cold water, and thus harden the paraffin as quickly as possible. Break the bottle carefully, cut off the lower portion of the paraffin containing the pollen, mount it on a block in the usual manner, and trim away some of the paraffin so that two parallel surfaces will make the sections ribbon well. Material in this stage shows a large tube nucleus, a somewhat lenticular cell with a more deeply staining nucleus, and, lastly, two small prothallial cells quite close to the spore wall. The prothallial cells cannot always be detected at this stage, and there may be some doubt as to whether two such cells are always present. The division of the lenticular cell into "stalk cell" and "body cell," and also the division of the body cell into the two male cells, must be looked for in sections of the nucellus of the ovule.

Oogenesis.—The entire ovulate cone at the time of pollination is easily cut in paraffin. Longitudinal sections of the cone at this time give good views of the bract and ovuliferous scale bearing the ovules. The integument is very well marked, and in the nucellus one or more sporogenous cells can usually be distinguished. As soon as the scales close up after pollination, the cone will be too hard to cut, and it will be necessary to remove the scales and cut them separately. For a study of the ovule and the structures within it, better preparations will be

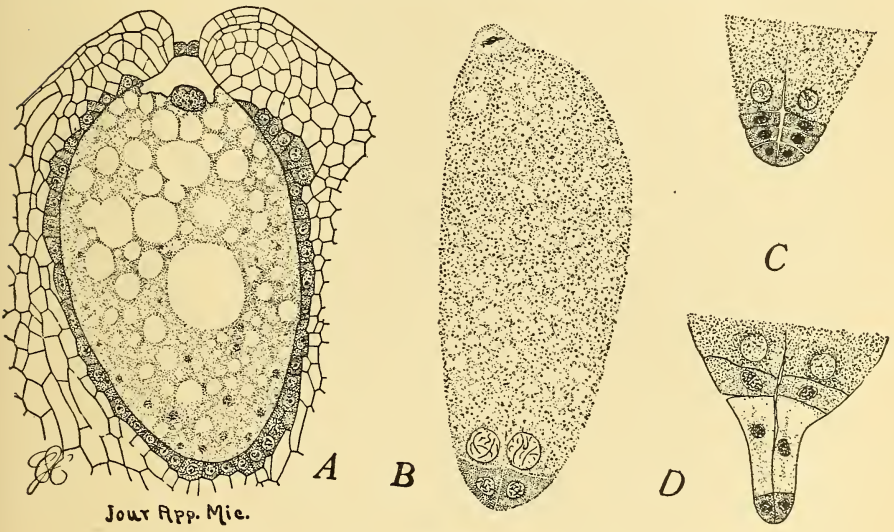

FIG. 60. Pinus Laricio. × 104.

A, top of prothallium with an archegonium just before the cutting off of the ventral canal cell. Fixed in Flemming's weaker solution and stained with Haidenhain's iron alum-hæmatoxylin. Collected June 18, 1897. *B*, *C*, and *D*, early stages in the formation of the embryo. Fixed in chromo-acetic acid, and stained in safranin-gentian violet-orange. Collected July 2, 1897.

obtained by carefully cutting off the pair of ovules from the scale. For preparations like that represented in *fig. 60, A*, it is a good plan to remove the endosperm with its archegonia from the ovule. Fixing, infiltration, and cutting will then occasion but little trouble, and the whole ribbon may be gotten upon a single slide. However, at this stage the pollen tubes with their contents are rapidly working their way through the nucellus toward the archegonia, and consequently it is better to retain

enough of the tissues of the ovule to keep the nucellus in place. In later stages, after fertilization has taken place, it is necessary to remove the endosperm. In stages like *fig. 60, B, C, D*, and later, the developing testa should be dissected away with great care, for a very slight pressure is sufficient to injure the delicate parts within. Mature embryos may be dissected out from the endosperm before fixing, but it is hardly necessary, since they cut quite well if left in place. The "pine nuts" or "piñon," to be found upon the market, are good for a study of the mature embryo. The testa, which is quite a hard shell, should be taken off, and the endosperm should be allowed to soak in water for about twenty-four hours, after which the embryo may be dissected out and fixed.

The period at which the various stages may be found varies with the species, the locality, and the season. In *Pinus Laricio* (the common Austrian pine) at Chicago, in the season of 1897, material collected May 27 did not yet show archegonia; the ventral canal cell was cut off about June 21 (see *fig. 61*), the fusion of the pronuclei occurred about a week later, and stages like *fig. 60, B, C*, and *D*, were common in material collected July 2. In the season of 1896 all the stages appeared about two weeks earlier. In *Pinus sylvestris* the stages appeared a little earlier than in *Pinus Laricio*. After the stage shown in *fig. 60, A*, has appeared, it is necessary to collect at intervals of not more than two days until the stage shown in *fig. 60, D*, is reached. If collections are made at intervals of four or five days, the most interesting stages, like the cutting off of the ventral canal cell, fertilization, and the first divisions of the nucleus of the oöspore, may be missed altogether. It should be mentioned that all the ovules of a cone will be in very nearly the same stage of development.

A rather strong chromo-acetic acid (1 g. chromic acid and ½ cc. glacial acetic acid to 100 cc. water) can be recommended for the entire series in spermatogenesis, oögenesis, fertilization, and formation of the embryo. After repeated trials the popular Flemming's solution does not seem to be at all

superior and often fails to give as good results as the cheaper fixing agent.

The following stains may be suggested: for studying the pollen tubes in the nucellus, cyanin and erythrosin; for the development of the archegonium up to the stage shown in *fig. 60, A*, Delafield's hæmatoxylin; for the stages shown in *fig. 60, A*, iron alum-hæmatoxylin or the safranin-gentian violet-orange combination; for the stage shown in *fig. 61*, nothing seems to equal the safranin-gentian violet-orange combination; for stages like *fig. 60, B, C*, and *D*, and also for later stages in the development of the embryo, Delafield's hæmatoxylin brings out the walls perfectly, but since mitotic figures are very frequent in these stages, it is worth while to use the safranin combination with some preparations,

FIG. 61. Pinus Laricio. × 710.

The mitotic figure concerned in cutting off the ventral canal cell. The nucleus at the lower end of the spindle is the nucleus of the oösphore. Fixed in chromo-acetic acid, and stained in the safranin-gentian violet-orange combination. Collected June 21, 1897.

although it is much inferior to Delafield's hæmatoxylin when cellulose walls are to be emphasized.

All the stages which have been described can be cut in paraffin with little difficulty.

The Leaves.—The leaves of our common gymnosperms cut readily in paraffin while they are young and tender, but as they approach maturity it is a fruitless task to attempt paraffin sections. Celloidin sections are far more satisfactory. Cut the needles into pieces about one-fourth of an inch long, fix in a picro-corrosive-acetic mixture (½ g. picric acid, 2 g. corrosive sublimate, 1 cc. glacial acetic acid, 100 cc. 50 per cent. alcohol). If used hot, five minutes is sufficient, but if used cold it should be allowed to act for two or three hours. After the material has been imbedded in celloidin, the block should be placed in equal parts of 95 per cent. alcohol and glycerine for a few days, after which it should cut quite readily. Stain with safranin and Delafield's hæmatoxylin, clear in Eycleshymer's clearing mixture, and mount in balsam.

Fairly good sections may be obtained in great quantities with little trouble by the following method: Make a bunch of the needles as large as one's little finger, wrap them firmly together with a string, allowing about an eighth of an inch of the bunch to project above the wrapping; then fasten the whole in a hand microtome, and every stroke of the razor will give twenty or thirty sections, some of which will surely be good. As the sections are cut, they may be put directly into 95 per cent. alcohol, and after a few minutes can be transferred to 50 per cent. alcohol and then to the stain. Dehydrate, clear in xylol, and mount in balsam.

Stems and Roots.—With a sharp razor fairly good sections of stems and roots may be made without imbedding, especially if the hand microtome be used. Young buds may be cut in paraffin. Stems and roots as large as half an inch in diameter can be cut in celloidin. The material should be cut into pieces not more than one-fourth of an inch long. The following treatment should give good results:

1. Picro-corrosive-acetic mixture, five minutes if used hot, or wo to three hours if used cold.

2. Wash in 50 per cent. alcohol, to which a little iodine has been added, two hours; 70 per cent., 85 per cent., 95 per cent., our hours each; absolute alcohol, ten hours; then change to fresh absolute alcohol, which should act for ten hours longer.

3. Ether alcohol, twenty-four hours. Some prefer to precede the ether alcohol by a mixture of equal parts of ether alcohol and absolute alcohol.

4. Thin celloidin (about 2 per cent.), two or three days; 6 per cent. celloidin, two or three days; 10 per cent. celloidin, two or three days. After the thin celloidin has acted for a few days, the cork may be removed for a short time each day, thus allowing the thin celloidin to become thick by the evaporation of the ether alcohol.

5. Get some small blocks of wood (three-eighths inch cubes of white pine are good), wet one of them in ether alcohol, dip it into thin celloidin, place the object upon the block in convenient position for cutting, pour over it a few drops of 10 per cent. celloidin, and then plunge the whole into chloroform. Leave it in the chloroform about twenty-four hours, and then transfer to a mixture of equal parts of 95 per cent. alcohol and glycerine, where it should remain for several days. Material may be kept here indefinitely. Even refractory stems may be cut after they have been in this mixture for a couple of weeks.

6. Cut the sections, keeping the knife wet with the alcohol and glycerine mixture. Transfer the sections to 70 per cent. alcohol, then to 50 per cent., and then to the stain.

7. Stain in safranin, twenty-four hours; wash in 35 per cent. alcohol, about a minute; stain in Delafield's hæmatoxylin, five to ten minutes; wash in water, two minutes; 35 per cent. alcohol, two to five minutes; 50 per cent. alcohol, two to five minutes; acid alcohol, one to ten seconds; 70 per cent., 85 per cent., 95 per cent., about two minutes each; Eycleshymer's clearing mixture until cleared, usually about one or two minutes. Mount in balsam.

Xylem should show a brilliant red color and cellulose a rich purple, if the stain is successful. If either stain is too weak or too prominent, the duration of the stain, the length of time in the alcohols, or the time in acid alcohol must be varied until the desired result is secured.

People who make all their anatomical sections without imbedding may regard this method as tedious and unnecessary, but such preparations will show much which is never seen in mere free-hand sections, for the reason that free-hand sections, if thin enough to show any detail, will lose most of their cell contents, while in celloidin sections everything is held in place. Even if the celloidin sections be passed through absolute alcohol and cleared with clove oil, a process which dissolves away the celloidin, the contents of the cells will still be retained in most cases, and stains which cannot be extracted from celloidin may be used. However, we prefer to use the safranin and Delafield's hæmatoxylin and retain the celloidin, since this combination can be extracted completely from the celloidin and still leave the object brilliantly stained.

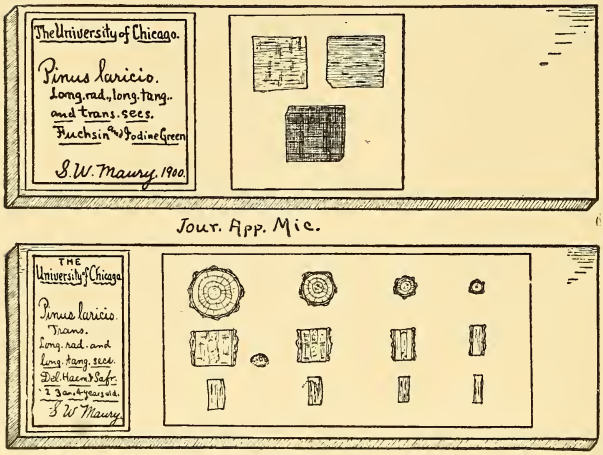

Fig. 62.

Slides showing labels and methods of arranging sections.

For preparations of the mature wood a piece of white pine from a dry-goods box furnishes perfect material. It should not be imbedded, but the cutting will be facilitated if the piece be soaked for a few hours in water or in the alcohol and glycerine mixture. In all preparations designed to show the structure of

wood there should be three sections — a transverse, a longitudinal radial, and a longitudinal tangential. These may be arranged as in the upper slide of *fig. 62*, but if it is desired to make a thorough study of the structure, it is a good plan to have on each slide several sections of each kind, thus having an opportunity to use a variety of stains. Fuchsin and iodine green is a good combination. The safranin and Delafield's hæmatoxylin is also excellent.

For a study of young stems or roots, preparations like that shown in the lower slide of *fig. 62* will be found very convenient.

CHAPTER XIX.

SPERMATOPHYTES.

ANGIOSPERMS.

Success depends largely upon judgment and care in selecting and trimming material before it is put into the fixing agent in the field. While the following directions cannot be applied to all plants, they should, nevertheless, enable the student to make such modifications as may be demanded by any particular form

Floral Development.— For a study of floral development very young buds are necessary, and it is best to select those forms which have rather dense clusters of flowers, in order that a complete series may be obtained with as little trouble as possible.

The usual order of appearance of floral parts is (1) calyx, (2) corolla, (3) stamens, and (4) carpels, but if any of these organs are reduced or metamorphosed, their order of appearance may be affected.

Floral development is easily studied in the common *Capsella bursa-pastoris*. The best time to collect material is late in March or early in April. Dig up the plant, carefully remove the leaves, and in the center of the rosette a tiny white axis will be found. A series of these axes from one-eighth of an inch to three-eighths of an inch in length and from one-sixteenth of an inch to three-sixteenths of an inch in diameter will give a very complete series of stages in the development of the floral organs. Preparations from the apex of the shoot taken after the inflorescence appears above ground are not to be compared with these taken early in the season. Fix in chromo-acetic acid and stain in Delafields hæmatoxylin. The sections should be longitudinal and about 5 μ thick.

The common dandelion, *Taraxacum officinale*, affords an excellent series with little labor. Examine vigorous plants which have, as yet, no flowers or buds in sight. Dig up the plant and dissect away the leaves. If there is a white cluster of flower buds, the

largest not more than three-sixteenths of an inch in diameter, cut out the cluster, leaving only enough tissue at the base to hold the buds in place. Larger heads should be cut separately. Fix and stain as in *Capsella*.

Our most common thistle, *Cnicus lanceolatus*, shows the floral development with unusual clearness, but the preparation of the material is somewhat tedious. The involucre, which is too hard to cut, must be carefully dissected away. Retain only enough of the receptacle to hold the developing florets in place. A series of sizes with discs varying from one-eighth of an inch to three-eighths of an inch in diameter will show the development from the undifferentiated papilla up to the appearance of the archesporial cell in the nucellus of the ovule. The Canada thistle, *Cnicus arvensis*, is equally good, but it is more difficult to dissect out the desirable parts.

In the willows, *Salix*, the bud scales must be removed and the copious hairs should be trimmed off as much as possible with scissors, after which the catkin should be cut in two longitudinally and placed in the fixing agent.

Spermatogenesis.—The earlier stages in spermatogenesis will be found in the preparations of floral development. For tracing the nuclear changes involved in this process the lilies furnish very good material, because the cells and nuclei are exceptionally large. Several species of *Lilium* are common in greenhouses, and these may be used where wild material is not available. In early stages where the sporogenous cells have not yet begun to round off into spore mother-cells it is sufficient to remove the perianth, retaining just enough of the receptacle to hold the stamens in place. Transverse sections show the six stamens and also the young ovary. After the spore mother-cells have begun to round off, each stamen should be removed so as to be cut separately. It is very desirable to secure stamens showing the mitotic figures which occur during the division of the spore mother-cell into the four microspores. Since the pollen mother-cells are apt to be in approximately the same stage of development throughout the anther, it is worth while to determine

whether mitotic figures are present before putting the material into the fixing agent. The procedure indicated for *Pinus* can be followed here. It is not necessary to cut the stamens into pieces before fixing, since they are easily penetrated and infiltrated; in later stages the stamens *must* not be cut into pieces, since the pollen grains are easily washed out.

Transverse sections are better for morphological purposes; but where nothing is desired except the development of pollen grains from the spore mother-cells, much more material can be gotten under a cover by using longitudinal sections.

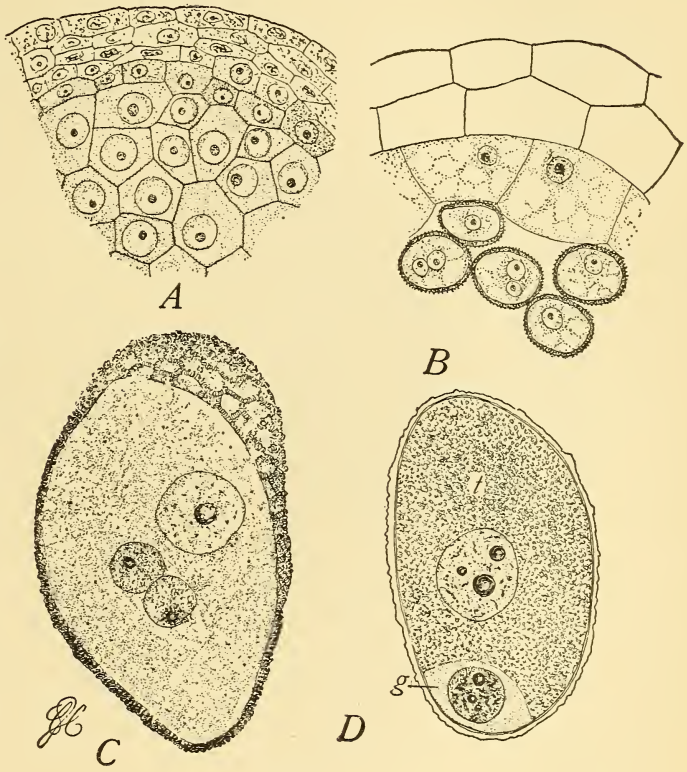

Fig. 63.

A, Salix tristis. × 594. Transverse section of a portion of a young anther showing large sporogenous cells, one layer of tapetal cells (more deeply shaded in the figure), and from three to four layers of wall cells. *B*, Salix petiolaris. × 594. Small portion of transverse section of a nearly mature anther showing five pollen grains, two tapetal cells, and two layers of wall cells. *C*, Lilium auratum. × 505. Section of a pollen grain showing the large tube nucleus and two smaller generative nuclei. Fixed in chromo-acetic acid and stained in safranin-gentian violet-orange. *D*, Lilium tigrinum. × 505. Pollen grain showing tube nucleus in the middle and a lenticular cell with the generative nucleus at one end of the grain.

For early stages up to that shown in *fig. 63*, *A*, Delafield's hæmatoxylin is very satisfactory; in stages like *B*, *C*, and *D* of the same figure, cyanin and erythrosin often give excellent differentiation, the tube nucleus taking the erythrosin, and the generative nucleus the cyanin, while the

starch grains which are often abundant at this stage take a faint pink from the erythrosin. However, the safranin-gentian violet-orange combination shows nuclear details to better advantage, and readily distinguishes the tube and generative nuclei, although it does not give such a striking color contrast as the cyanin and erythrosin. The starch grains are brilliantly stained by the gentian violet.

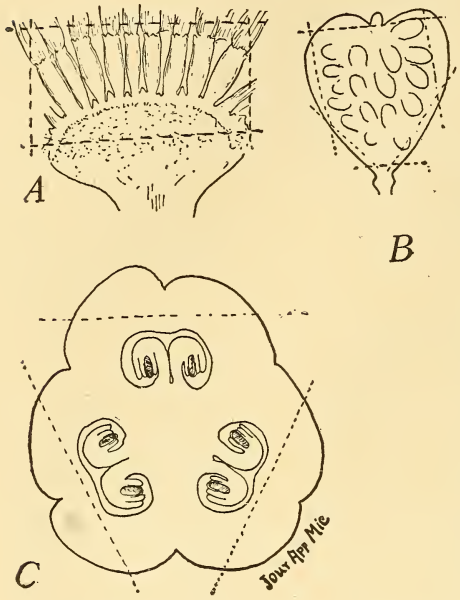

FIG. 64.

A, head of Aster. *B*, pod of Capsella. *C*, transverse section of ovary of Lilium. The dotted lines show how the material should be trimmed before fixing.

The mature pollen grain, after shedding, may be prepared by the method already described for *Pinus*.

Oögenesis. — As in spermatogenesis, the early stages will be found in preparations of floral development. The origin and development of the macrospore are easily traced in *Lilium*. In very young stages, before the appearance of the integument, the ovary may be removed from the flower and placed directly in the fixing agent, but in later stages, such as are shown in *figs. 66–71*, strips should be cut off from the sides of the ovary in order to secure more rapid fixing and more perfect infiltration with paraffin. The dotted lines in *fig. 64, C*, show about how much should be cut off. This is a much better plan than to secure rapid fixing and infiltration by cutting the ovary into short pieces, because the ovules will be in about the same stage of development throughout the ovary, and when one finds desirable stages like those from which these photomicrographs were taken, it is gratifying to have these pieces as long as possible.

In lilies, and other forms with large cells, the entire series shown in *figs. 65–70* may be fixed in chromo-acetic acid and

stained in safranin-gentian violet-orange, but stages earlier than that shown in *fig. 65* are more satisfactory if stained in Delafield's hæmatoxylin. Stages like that shown in *fig. 66* and also those between *fig. 66* and *fig. 67* are the most difficult to stain, and only the utmost care and patience will insure first-class preparations. The thread on the nucleus in *fig. 66* shows a row of chromatin granules and soon becomes segmented into twelve chromosomes. If the stain is too dense, the thread will probably appear smooth throughout all these stages, but if the staining is successful, the granules are sharply stained by the gentian violet, while the linin thread is stained lightly or not at all. In successful staining with cyanin and erythrosin the granules stain blue and the linin red. Stages like *figs. 67* and *68* are easily stained, and the preparations are exceptionally beautiful. Stain for twenty-four hours in safranin (the solution in 50 per cent. alcohol is very good), and then rinse in 50 per cent. alcohol until the red color disappears from the spindle, but remains bright in the chromosomes and nucleoli; stain in gentian violet four to eight minutes; rinse in water about thirty seconds; stain in aqueous orange G fifteen to thirty seconds; transfer to absolute alcohol, and move the slide gently back and forth in order to dehydrate as rapidly as possible (three to six seconds will usually be long enough); the slide must be taken from the absolute alcohol while the gentian violet is still coming out in streams; treat with clove oil ten to thirty seconds, and then drain off the clove oil and add a few drops of cedar oil, since the gentian violet fades if much clove oil is left in the preparation. If the cedar oil is

FIG. 65. Lilium philadelphicum. × 710.

Apex of nucellus contains a large archesporial cell with a large nucleus. In Lilium this archesporial cell becomes the embryo-sac directly without cutting off any tapetal cell or dividing into potential megaspores. Fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin.

transparent and has a strong odor, do not use it, but remove as much of the clove oil as possible. Mount directly in balsam. Before transferring to absolute alcohol, a single dip in 95 per cent. alcohol may do no damage and is a matter of economy, since it avoids carrying so much water into the absolute alcohol.

FIG. 66. Lilium philadelphicum. × 710.

Nucellus with megaspore. The chromatin thread in the nucleus is very distinct. Fixed in chromo-acetic acid and stained in safranin-gentian violet-orange.

When the staining is properly done, the chromosomes will show a bright red color and the spindle a brilliant violet. If the action of the gentian violet be too prolonged or poorly extracted, the chromosomes will appear violet or reddish violet, and such details as the relation of spindle fibers to chromosomes will be obscured. Stages shown in *figs.* *69* and *70* may be found in the same ribbons with stages like *figs.* *67* and *68*, and are well differentiated by the same treatment.

Later stages up to fertilization and the first divisions of the embryo may be fixed and stained as already described, but

cyanin and erythrosin gives a particularly brilliant effect, especially after Carnoy's fluid. At the stage shown in *fig. 71*, the male nucleus takes the cyanin, and the oösphere nucleus the erythrosin, although at a slightly later stage they stain alike.

FIG. 67. Lilium philadelphicum. × 710. FIG. 68.

First division of the nucleus of the megaspore. (The same nucleus the earlier stages of which are shown in figs. 65 and 66.) Fixed in chromo-acetic acid, and stained in safranin-gentian violet-orange. Fifteen microns.

For the later stages in the development of the embryo, Delafield's hæmatoxylin is a far better stain.

Many of the Compositæ, like *Aster*, *Taraxacum*, *Senecio*, and *Silphium*, are excellent for a study of the mature sac and the formation of the embryo. The whole head may be cut if trimmed as indicated in *fig. 64*, but for the embryo-sac at the fertilization period, and also for the development of the embryo, it is worth while to resort to the tedious process of dissecting the ovules

out from the ovaries. Stages like those shown in *figs. 65–70* are rather unsatisfactory in Composites.

Many of the Ranunculaceæ are easily studied. *Anemone patens*, var. *Nuttalliana*, has a very beautiful embryo-sac, the egg,

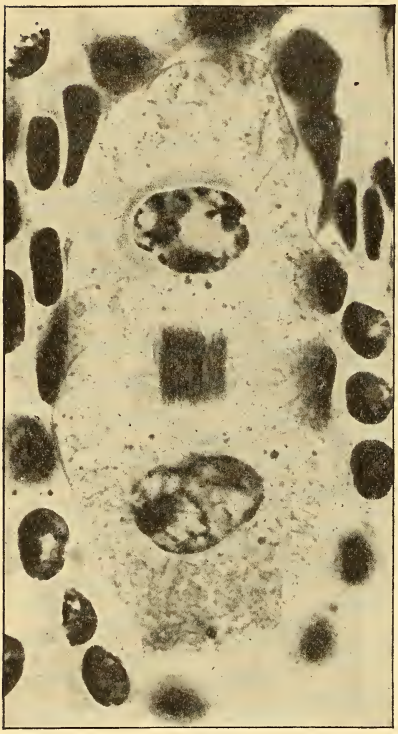

FIG. 69. Lilium philadelphicum. × 710.

Megaspore (embryo-sac) containing two daughter-nuclei resulting from the first division of the nucleus of the megaspore. A portion of the spindle still remains between the two nuclei. Fixed in chromo-acetic acid, and stained in safranin-gentian violet-orange. Fifteen microns thick.

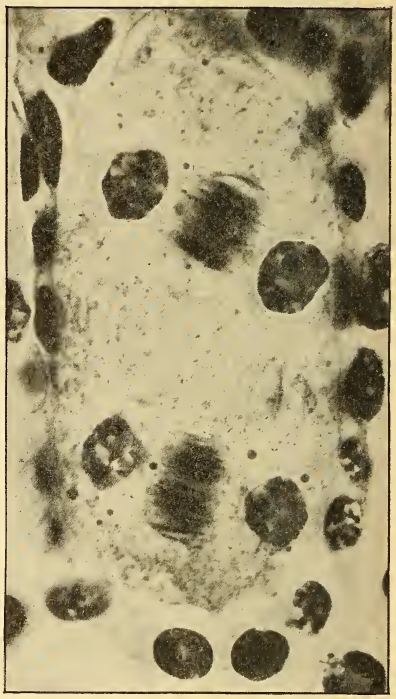

FIG. 70. Lilium philadelphicum. × 710.

Later stage in the development of the embryo-sac. Each of the two nuclei shown in fig. 69 has divided. The nuclei are much smaller than those in fig. 69. Fixed in chromo-acetic acid, and stained in safranin-gentian violet-orange. Fifteen microns thick.

synergids, endosperm nucleus, and antipodals being rather large and sharply defined. *Hepatica*, *Caltha*, and some species of *Ranunculus* are exceptionally good.

Development of the Embryo.— The common shepherd's purse (*Capsella bursa-pastoris*) is a favorable form for a study of the development of a dicotyl embryo. The stages shown in *fig. 72*,

A–F, will be found in pods which are about one-eighth of an inch long. These may be put directly into the fixing agent, but stages like *G* and *H* are found in pods about three-sixteenths of an inch long, and such stages will be more readily fixed, infiltrated, and cut if the pods are trimmed, as shown in *fig. 64, B*, before putting them into the fixing agent. Cut sections parallel to the flat face of the pod. Delafield's hæmatoxylin, without any contrast stain, gives the best results which we have secured.

For tracing the development of a monocotyl embryo *Sagittaria variabilis* can be recommended. Material is abundant, sections are easily cut, except in the latest stages, and it is not difficult to get a complete series. *Alisma plantago*, which is commonly figured in text-books, is extremely hard to cut, especially in later stages.

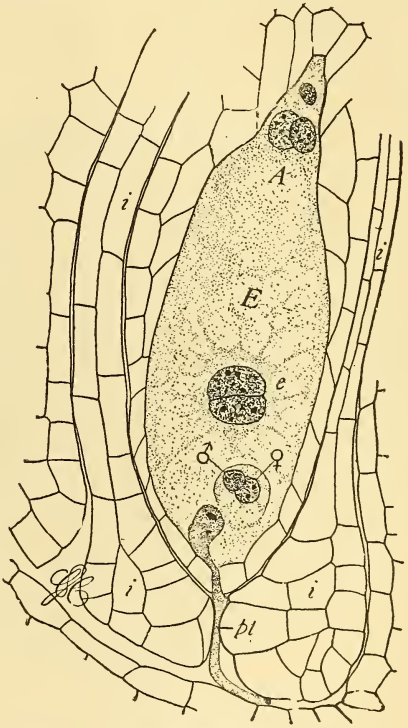

FIG. 71. Lilium philadelphicum. × 335.

Embryo-sac at time of fertilization. *A*, the three antipodals. *E*, protoplasm of the sac. *e*, polar nuclei fusing to form the endosperm nucleus; the male nucleus is about to fuse with the nucleus of the oösphere. *i*, the inner integument. *pt*, the pollen tube. Fixed in Carnoy's fluid and stained in cyanin and erythrosin. Fifteen microns thick. (From Bailey's Cyclopedia of American Horticulture.)

Leaves.—Where only a rapid examination is to be made, free-hand sections may be made in great numbers by using the method employed for pine needles. It is easy to get good sections of leaves which can be gotten into paraffin, but it is difficult to get tender, succulent leaves into paraffin without distortion. Such leaves may be cut in celloidin. For a study of the stomata, strip a piece of epidermis from the leaf, fix it, stain in Delafield's hæmatoxylin and erythrosin, pass it gradually through the alcohols, clear in xylol, and mount in balsam. Lily, tulip, hyacinth, and begonia may be suggested as favorable forms. Epidermis from the leaf

of the common *Sedum pupurascens* will usually show stomata in all stages of development.

Stems and Roots.—The earlier stages in the development of vascular bundles in stems and roots are well shown in paraffin

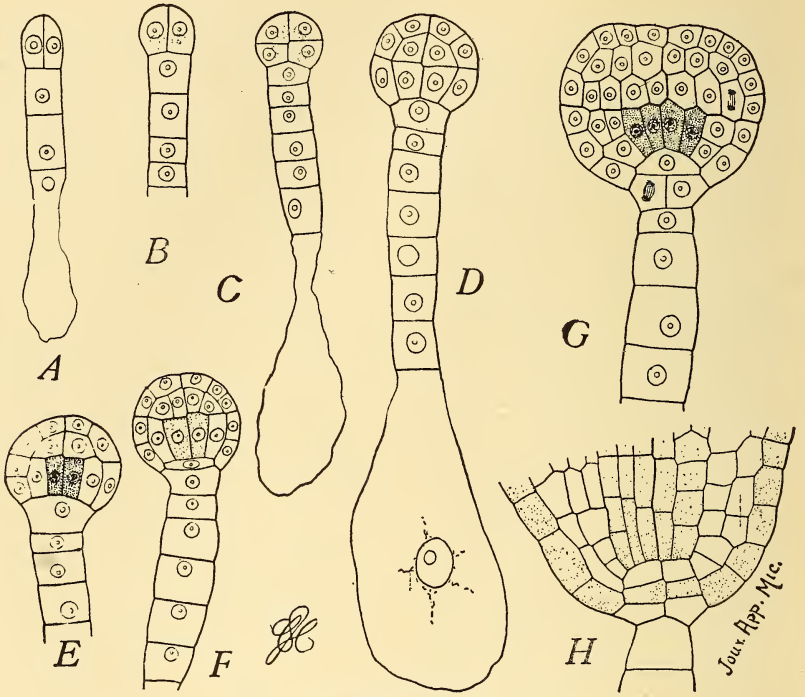

FIG. 72. Capsella bursa-pastoris. × 400.

A, first division in the embryo cell. *B*, quadrants. *C*, octants. *D*, the dermatogen has been cut off. There are eight cells in the suspensor, the lower cell being very large and vesicular. *E*, differentiation in plerome and periblem. The plerome cells are shaded. *F*, the periblem of the root is completed at the expense of the upper cell of the suspensor. *G*, the mitotic figure in the suspensor cell indicates that the upper suspensor cell by a second contribution is about to complete the dermatogen of the root. *H*, plerome (shaded), periblem, dermatogen (shaded), and first layer of the root cap. Fixed in chromo-acetic acid and stained in Delafield's hæmatoxylin. Ten microns thick.

sections of young seedlings. The common bean is a favorable form, and it is easy to get material.

Most herbaceous stems and roots, and also the younger woody stems and roots, give the best results when cut in celloidin, as already described for *Pinus*. *Rumex crispus* and *Ranunculus repens* can be recommended for a study of the vascular bundles. The cambium is very sharply brought out by Delafield's

hæmatoxylin. Petioles or leaf blades of *Nuphar* imbedded in celloidin, and stained in safranin and Delafield's hæmatoxylin, yield extremely beautiful preparations, the sclerotic cells taking a brilliant red and the cellulose a rich purple. The sections should be 20–30 μ thick.

Stems or petioles of the squash or pumpkin are to be preferred for demonstrating sieve tubes and companion cells. For the more minute details of the sieve plate it is best to cut out small pieces about one-fourth of an inch long and one-eighth of an inch square containing the vascular bundle. These pieces can be imbedded in paraffin.

For demonstrating the phellogen and the tissue developed from it, stems of *Geranium* or *Coleus* about one-fourth of an inch in diameter or seedlings of *Xanthium canadense* about three-sixteenths of an inch in diameter can be recommended. They can be cut in paraffin, but satisfactory results are more uniformly obtained from celloidin sections.

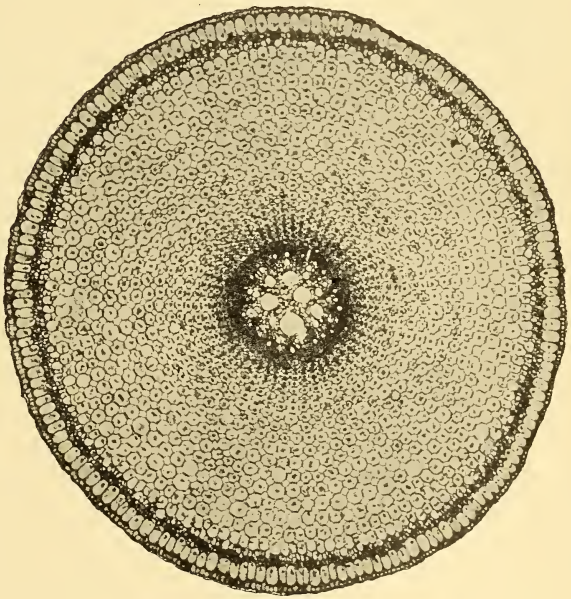

FIG. 73. Sparganium eurycarpum. × 53.
Tranverse section of a root. Delafield's hæmatoxylin and acid fuchsin. Five microns.

The stem of Indian corn, imbedded in celloidin and stained in safranin and Delafield's hæmatoxylin, affords a good study of monocotyl stem anatomy.

It must not be forgotten that root tips, besides showing anatomical structure, furnish ever-ready material for the study of karyokinesis. An onion thrown into a pan of water will soon

send out numerous roots. About one-fourth of an inch should be cut off from the tip of the root. Fix and stain as directed for the mitotic figures in *Lilium*. While the onion is very available material, the figures are not as satisfactory as those to be obtained in the root tips of *Tradescantia virginica*, *Iris versicolor*, *Podyphyllum peltatum*, *Arisæma triphyllum*, *Cypripedium pubescens*, and many others. Transverse sections of young roots often show a remarkably regular arrangement of the cells, as can be seen in *fig. 73*.

We are painfully aware that the directions which have been given in this series of articles are very incomplete, but it is hoped that they will enable the student to devise for himself such methods as particular cases may demand.

CHAPTER XX.

LABELING AND CATALOGUING PREPARATIONS.

THE LABEL.

The labels shown in *fig. 62*, on p. 126, show as much as will generally be found desirable. The date of the collection of the material is often needed in addition. The date of making the preparation is of no value unless the student is testing the permanence of stains or something of that sort. It is hardly worth while to write upon the label the names of the stains used, for the student will soon learn to recognize the principal stains. We should say that the *first* thing to write upon a label is the genus and species of the plant; the next thing would be the name of the organ or tissue, and then might be added the date of collection: *e. g.*, *Marchantia polymorpha*, young archegonia, April 10, 1901. A hasty sketch on the label will often indicate any exceptionally interesting feature in the preparation. To facilitate finding such a feature, it is a good plan to mark the particular section or sections with ink, the marking being always on the underside of the slide so as not to cause any inconvenience if an immersion lens should be used.

CATALOGUING PREPARATIONS.

As a collection grows, the student will need some device for readily locating any particular preparation. Some have their slides numbered and catalogued, but all devices of this sort are too cumbrous and slow for the practical worker in the laboratory. After several years' experience with a collection which now numbers about seven thousand preparations, the following method can be confidently recommended:

Four wooden slide boxes of the usual type will do for a beginning; they should be labeled: THALLOPHYTA, BRYOPHYTA, PTERIDOPHYTA, and SPERMATOPHYTA. As the collection grows

and new boxes are needed, the classification can be made more definite; *e.g.*, there should be a box labeled BRYOPHYTES *Hepaticæ* and one labeled BRYOPHYTES *Musci*. As the liverwort collection grows, three boxes will be necessary, and should be labeled BRYOPHYTES *Hepaticæ* Marchantiales, BRYOPHYTES *Hepaticæ* Jungermanniales, and BRYOPHYTES *Hepaticæ* Anthocerotales. It will readily be seen that the process can be continued almost indefinitely, and that new slides may be at any time dropped into their proper places. A rather complete label gradually built up in this way is shown in *fig. 74:*

BRYOPHYTES
HEPATICÆ
Jungermanniales
Porella platyphyllum
Archegonia

FIG. 74.

CHAPTER XXI.

A CLASS LIST OF PREPARATIONS.

Where a regular course in histology is conducted, it is a good plan to give each student at the outset a complete list of the preparations which he is expected to make. In a three-months' course a fairly representative collection of preparations can be made. The availability of material determines what a list shall be. The following list was recently used by one of the writer's classes:

LIST OF PREPARATIONS.

THALLOPHYTES.

ALGÆ.

CYANOPHYCEÆ.

1. *Wasserblüthe.*—The principal forms in this material are:
   (*a*) *Cœlosphærium Kützingianum.* — Colonies in the form of hollow spheres.
   (*b*) *Anabæna gigantea.*— Filaments straight. Preparations should show vegetative cells, heterocysts, hormogonia, and spores.
   (*c*) *Anabæna flos-aquæ.*— Filaments curved. Stain on the slide and mount in balsam.
2. *Nostoc.*—Eosin or erythrosin. Glycerine.
3. *Oscillaria.*— Put living material into 10 per cent. glycerine and allow it to concentrate.
4. *Glœotrichia.*— Eosin, erythrosin, or iron alum-hæmatoxylin. Glycerine. Should show heterocysts, spores, and vegetative filaments.
5. *Tolypothrix.*—Treat like *Oscillaria* or *Gloeotrichia.* Should show heterocysts, hormogonia, and false branching.

CHLOROPHYCEÆ.

6. *Volvox.*— Stain some in eosin or erythrosin and some in very dilute Delafield's hæmatoxylin. Mount in glycerine in a cell deep enough to prevent crushing. Try to have antheridia, oögonia, and young colonies in each mount.
7. *Vaucheria.*— Eosin, erythrosin, or iron alum-hæmatoxylin. Glycerine. Should show antheridia, oögonia, zoöspores.

8. *Hydrodictyon.*— Iron alum-hæmatoxylin or dilute Delafield's hæmatoxylin. Glycerine. Look for young nets inside the older segments.
9. *Spirogyra.*— Iron alum-hæmatoxylin. A counter-stain with eosin is a great improvement, if successful. Glycerine. Should show chromatophores, pyrenoids, and nucleus. Eosin, erythrosin, or dilute Delafield's hæmatoxylin will be better for conjugating material.
10. *Zygnema.*— Same treatment as for *Spirogyra* to bring out chromatophores, pyrenoids, and nucleus. Use iron alum-hæmatoxylin for reproductive stages.
11. *Diatoms.*— Living and fossil forms. Balsam.
12. *Oedogonium.*— Use iron alum-hæmatoxylin for oögonia, antheridia, and zoöspores. Eosin or erythrosin for caps and other vegetative structures. Glycerine.
13. *Chara.*— Oögonia and antheridia may be mounted whole in a cell deep enough to prevent crushing. Glycerine jelly. Paraffin sections of oögonia, antheridia, and apical cell.

PHÆOPHYCEÆ.

14. *Ectocarpus.*— Stain some in eosin and some in dilute Delafield's hæmatoxylin. Glycerine. Each mount should show both unilocular and multilocular sporangia.
15. *Fucus vesiculosus.*— Antheridial conceptacle with paraphyses and antheridia; oögonial conceptacle with oögonia. Delafield's hæmatoxylin for paraffin sections. Borax carmine for teased preparations.

RHODOPHYCEÆ.

16. *Batrachospermum* (or *Nemalion*).— Mayer's hæm-alum. Glycerine. Should show trichogyne, carpogonium, and cystocarp.
17. *Polysiphonia fibrillosa.*— Iron alum-hæmatoxylin or eosin. Glycerine. Vegetative structure, tetraspores, cystocarps with carpospores, antheridia.

FUNGI.

MYXOMYCETES.

18. *Trichia varia.*— Paraffin sections, 5 μ. Safranin-gentian violet-orange.

SCHIZOMYCETES.

19. *Bacteria.*— *Coccus*, *Bacillus*, and *Spirillum* forms. Stain on cover-glass or slide.
20. *Bacillus anthracis.*— In liver of mouse. Paraffin sections, 5 μ. Stain in gentian violet, Gram's method.

PHYCOMYCETES.

21. *Mucor stolonifer.*— Stain young sporangia in eosin or dilute Delafield's hæmatoxylin. Zygosporic material may be mounted without staining or after a *very* light staining in dilute Delafield's hæmatoxylin. Glycerine.

22. *Cystopus candidus* on *Cakile americana*.— Select white blisters which have not yet broken open. Paraffin, 5 μ. Safranin-gentian violet-orange.
23. *Cystopus bliti* on *Amarantus retroflexus*.— Cut out small portions of leaves in which the oögonia can be seen in abundance. Paraffin, 5 μ.

ASCOMYCETES.

24. *Eurotium*.— Eosin or erythrosin. Glycerine.
25. *Pencillium*.— Eosin or erythrosin. Glycerine.
26. *Erysiphe commune* on *Polygonum aviculare*.— Strip the fungus from the leaf. Paraffin, 5 μ. Safranin-gentian violet-orange.
27. *Uncinula necator* on *Ampelopsis quinquefolia*.— Eosin or erythrosin. Mount whole and break the perithecia under the cover. Glycerine or balsam.
28. *Xylaria*.— Paraffin sections of younger stages. Delafield's hæmatoxylin and erythrosin. Be sure that some section in each mount shows the opening of a perithecium.
29. *Peziza*.— Paraffin sections of young apothecia, 5 μ or less; sections of older apothecia, 10 μ or 15 μ. Safranin-gentian violet-orange.

ÆCIDIOMYCETES.

30. *Puccinia graminis*.— Æcidium stage on barberry leaf. Paraffin. Cyanin and erythrosin. Uredospore and teleutospore stage in celloidin or paraffin.

BASIDIOMYCETES.

31. *Coprinus comatus*.— Paraffin. Transverse sections of gills showing trama, paraphyses, basidia, and spores. To show the basidium with four spores, the sections should be 15 μ thick. For development of the spores, cut 5 μ or less. Safranin-gentian violet-orange. *Boletus*, *Hydnum*, and *Polyporus* are treated in the same manner.

LICHENS.

32. *Parmelia Borreri*.— Paraffin, 5 μ. Cyanin and erythrosin.

BRYOPHYTES.

HEPATICÆ.

33. *Ricciocarpus natans*.— Paraffin, 10 μ or 15 μ. Delafield's hæmatoxylin. Archegonia, antheridia, and sporophytes imbedded in the gametophyte.
34. *Marchantia polymorpha*.— Paraffin, 10 μ. Archegonia, antheridia, and sporophytes.
35. *Pellia epiphylla*.— Paraffin, 10 μ. Longitudinal sections of sporophyte attached to gametophyte. Delafield's hæmatoxylin and erythrosin.
36. *Porella platyphyllum*.— Paraffin, 10 μ. Delafield's hæmatoxylin. Archegonia, antheridia, sporophyte, and apical cell.
37. *Anthoceros lævis*.— Paraffin, 5 or 10 μ. Longitudinal and transverse sections of sporophyte. Safranin-gentian violet-orange.

MUSCI.

38. *Sphagnum.*— Leaf buds. Safranin and Delafield's hæmatoxylin. Tease and mount in balsam.
39. *Sphagnum.*— Capsule. Paraffin. Delafield's hæmatoxylin and erythrosin.
40. *Bryum proliferum.*— Paraffin. Antheridia, 10 μ; archegonia, 15 to 20 μ; capsule, 10 μ.
41. *Funaria hygrometrica.*— Paraffin. Longitudinal and transverse sections of young capsules. Delafield's hæmatoxylin.
42. *Funaria hygrometrica* or any favorable form. Protonema. Place the well-cleaned material directly into 10 per cent. glycerine and allow it to concentrate.

PTERIDOPHYTES.

FILICALES.

43. *Botrychium virginianum.*— Paraffin. Stain rhizome, stipe, and root in safranin and Delafield's hæmatoxylin. Stain sporangia in Delafield's hæmatoxylin.
44. *Isoetes echinospora.*— Transverse section of stem. Celloidin. Safranin and Delafield's hæmatoxylin.
45. *Isoetes echinospora.*— Paraffin. Longitudinal sections of microsporangia and megasporangia. Safranin-gentian violet-orange.
46. *Pteris aquilina.*— Free-hand sections. Safranin and Delafield's hæmatoxylin, or iodine green and fuchsin. Balsam.
47. *Pteris cretica.*— Paraffin. Transverse sections of leaves with sporangia. Delafield's hæmatoxylin and erythrosin or safranin-gentian violet-orange.
48. *Adiantum cuneatum.*— Prothallia mounted whole in glycerine. Paraffin sections of antheridia and archegonia.
49. *Marsilea quadrifolia.*— Paraffin. Longitudinal and transverse sections of sori. Safranin-gentian violet-orange.
50. *Azolla carolinense.*— Paraffin. Vertical sections of the whole plant showing micro- and megasporangia.

EQUISETALES.

51. *Equisetum arvense.*— Prothallia in glycerine. Stem tips in paraffin. Transverse section of stem in celloidin.

LYCOPODIALES.

52. *Lycopodium lucidulum.*— Transverse section of stem. Free-hand or in celloidin. Safranin and Delafield's hæmatoxylin.
53. *Lycopodium inundatum.*— Paraffin. Longitudinal sections of strobilus.
54. *Selaginella.*— Paraffin. Longitudinal sections of rather mature strobili. Cyanin and erythrosin.

SPERMATOPHYTES.

GYMNOSPERMS.

55. *Pinus Laricio.*—Transverse section of needles and young stem. Free-hand or celloidin. Safranin and Delafield's hæmatoxylin.
56. *Pinus strobus.*—Free-hand sections of well-seasoned wood. Iodine green and fuchsin, or safranin and Delafield's hæmatoxylin.
57. *Pinus Laricio.*—Paraffin. Ovule with archegonia. Safranin-gentian violet-orange.
58. *Pinus sylvestris* or *P. Laricio.*—Paraffin. Embryos.
59. *Pinus Laricio.*—Paraffin. Longitudinal section of mature staminate strobilus. Safranin-gentian violet-orange.

ANGIOSPERMS.

MONOCOTYLS.

60. *Lilium philadelphicum.*—Paraffin, 10 μ. Transverse sections of ovary; transverse sections of anthers.
61. *Tradescantia virginica.*—Paraffin, 5 and 10 μ. Longitudinal sections of root. Stain for mitosis.
62. *Allium.*—Paraffin. Transverse sections of older roots for vascular system.
63. *Lilium longiflorum.*—Strip epidermis from leaf and stain for stomata. Cut sections of leaf in paraffin, 10 to 20 μ.
64. *Zea mais.*—Celloidin. Longitudinal and transverse sections of stem.

DICOTYLS.

65. *Nuphar advena.*—Celloidin. Section of leaf 15 to 40 μ thick. Safranin and Delafield's hæmatoxylin.
66. *Capsella bursa-pastoris.*—Paraffin. Floral development, 5 μ. Embryos, 5 to 10 μ. Stain both in Delafield's hæmatoxylin without any contrast stain.
67. *Taraxacum officinale.*—Paraffin. Floral development, 5 μ. Embryo-sac, 10 to 15 μ.
68. *Anemone patens.*—Paraffin. Embryo-sac.
69. *Smilax herbacea.*—Celloidin. Transverse section of root. Safranin and Delafield's hæmatoxylin.
70. *Xanthium canadense.*—Celloidin. Transverse section of stem of seedling to show cambium and phellogen.
71. *Tilia americana.*—Celloidin or free-hand. Transverse sections of small stems one-eighth to one-fourth of an inch in diameter. Safranin and Delafield's hæmatoxylin.

In making the mounts the order indicated in the list should not be followed. Begin with free-hand sections, then study the

glycerine method, and then devote a large portion of the time to the paraffin method. Let the celloidin method come last.

It is neither possible nor desirable that each student should in every case go through all the processes from collecting material to labeling. Some of the material may be in 70 per cent. alcohol, some in formalin, some in glycerine, and some in paraffin. One student may imbed in paraffin enough of the *Anemone* for the whole class, another may imbed the *Lilium* stamens, and by such a division of labor a great variety of preparations may be secured without a corresponding demand upon the time of the individual.

CHAPTER XXII.

FORMULÆ FOR REAGENTS.

FIXING AGENTS.

Carnoy's Fluid.—

Absolute alcohol, 6 parts.
Chloroform, 3 parts.
Glacial acetic acid, 1 part.

Strong Chromo-Acetic Solution.—

Chromic acid, 1 g.
Glacial acetic acid, 1 cc.
Water, 98 cc.

Weak Chromo-Acetic Solution (Schaffner's).—

Chromic acid, 0.3 g.
Glacial acetic acid, 0.7 cc.
Water, 99 cc.

Medium Chromo-Acetic Solution.—

Chromic acid, 0.7 g.
Glacial acetic acid, 0.5 cc.
Water, 100 cc.

Flemming's Fluid (weaker solution).—

A { 1 per cent. chromic acid, 25 cc.
  { 1 per cent. acetic acid, 10 cc.
  { Water, 55 cc.

B. 1 per cent. osmic acid, 55 cc.

Keep the mixture A made up, and add B as the reagent is needed for use, since it does not keep well.

Flemming's Fluid (stronger solution).—

1 per cent. chromic acid, 45 cc.
2 per cent. osmic acid, 12 cc.
Glacial acetic acid, 3 cc.

Merkel's Fluid.—

1.4 per cent. solution of chromic acid, 25 cc.
1.4 per cent. solution of platinic chloride, 25 cc.

Hermann's Fluid.—

1 per cent. platinic chloride, 15 parts.
Glacial acetic acid, 1 part.
2 per cent. osmic aid, 4 or 2 parts.

Picric Acid.—

Picric acid, 1 g.
Water, or 70 per cent. alcohol, 100 cc.

Corrosive Sublimate.—

Bichloride of mercury, 4 g.
Glacial acetic acid, 2 cc.
Water, or 70 per cent. alcohol, 100 cc.

Formalin (weaker solution).—

Formalin, 2 cc.
Water, 98 cc.

Formalin (stronger solution).—

Formalin, 4 cc.
Water, 100 cc.

Osmic Acid.—

Osmic acid, 1 cc.
Distilled water, 100 cc.

The bottle in which the solution is to be kept, and also the glass tube in which the acid is sold, must be thoroughly cleaned. Break off the end of the tube, and drop both tube and acid into the distilled water.

STAINS.

Delafield's Hæmatoxylin.—"To 100 cc. of a saturated solution of ammonia alum add, drop by drop, a solution of 1 g. of hæmatoxylin dissolved in 6 cc. of absolute alcohol. Expose to air and light for one week. Filter. Add 25 cc. of glycerine and 25 cc. of methyl alcohol. Allow to stand until the color is sufficiently dark. Filter and keep in a tightly stoppered bottle." (Stirling and Lee.)

The solution should stand for at least two months before it is ready for using.

Erlich's Hæmatoxylin.—

Distilled water, 50 cc.
Absolute alcohol, 50 cc.
Glycerine, 50 cc.
Glacial acetic acid, 5 cc.
Hæmatoxylin, 1 g.
Alum in excess.

Keep it in a dark place until the color becomes a deep red. If well stoppered, it will keep indefinitely.

Boehmer's Hæmatoxylin.—

A { Hæmatoxylin, 1 g.
  { Absolute alcohol, 12 cc.

B { Alum, 1 g.
  { Distilled water, 240 cc.

The solution A must ripen for two months. When wanted for use, add about 10 drops of A to 10 cc. of B. Stain ten to twenty minutes. Wash in water and proceed as usual.

Mayer's Hæm-Alum.—Hæmatoxylin, 1 g., dissolved with heat in 50 cc. of 95 per cent. alcohol and added to a solution of 50 g. of alum in a liter of distilled water. Allow the mixture to cool and settle; filter; add a crystal of thymol to preserve from mold. (Lee.)

It is ready for use as soon as made up. Unless attacked by mold, it keeps indefinitely.

Haidenhain's Iron Alum-Hæmatoxylin.—This stain was introduced by Haidenhain in 1892 and has gained a well-deserved popularity with those engaged in cytological work. Two solutions are used, and they are never mixed:

A. One and one-half to 4 per cent. aqueous solution of ammonia sulphate of iron. (At present we use a 3 per cent. solution.)

B. One-half per cent. aqueous solution of hæmatoxylin.

Greenacher's Borax Carmine.—

Carmine, 3 g.
Borax, 4 g.
Distilled water, 100 cc.

Dissolve the borax in water and add the carmine, which is quickly dissolved with the aid of gentle heat. Add 100 cc. of 70 per cent. alcohol and filter. (Stirling.)

Alum Carmine.—A 4 per cent. aqueous solution of ammonia alum is boiled twenty minutes with 1 per cent. of powdered carmine. Filter after it cools. (Lee.)

Alum Cochineal.—

Powdered cochineal, 50 g.
Alum, 5 g.
Distilled water, 500 cc.

Dissolve the alum in water, add the cochineal, and boil; evaporate down to two-thirds of the original volume, and filter. Add a few drops of carbolic acid to prevent mold. (Stirling.)

Picro-Carmine.—

Picro-carmine (picro-carminate of ammonia), 1 g.
Water, 100 cc.

Eosin.—

Eosin, 1 g.
Water, or 70 per cent. alcohol, 100 cc.

General Formula for Anilins.—Make a 3 per cent. solution of anilin oil in distilled water; shake well and frequently for a day; add enough alcohol to make the whole mixture about 20 per cent. alcohol; add 1 g. of cyanin, erythrosin, safranin, gentian violet, etc., to each 100 cc. of this solution.

Iodine Green.—

Iodine green, 1 g.
70 per cent. alcohol, 100 cc.

Methyl Green.—

Methyl green, 1 g.
Glacial acetic acid, 1 cc.
Water, 100 cc.

If the preparation is to be mounted in balsam, a slight trace of acetic acid and also a trace of methyl green should be added to the absolute alcohol used for dehydrating.

Fuchsin.—

Fuchsin, 1 g.
95 per cent. alcohol, 100 cc.
Water, 100 cc.

Ziehl's Carbol Fuchsin.—

Fuchsin, 1 g.
Carbolic-acid crystals, 5 g.
95 per cent alcohol, 10 cc.
Water, 100 cc.

Fuchsin and Iodine Green Mixtures.—Two solutions are kept separate, since they do not retain their efficiency long after they are mixed.

A { 0.1 g. fuchsin (acid).
  { 50 cc. distilled water.

B { 0.1 g. iodine green.
  { 50 cc. distilled water.

C { 100 cc. absolute alcohol.
  { 1 cc. glacial acetic acid.
  { 0.1 g. iodine.

Stain in equal parts of A and B. Transfer from the stain directly to solution C and from C to xylol.

Another Formula.—

A { Acid fuchsin, 0.5 g.
  { Water, 100 cc.

B { Iodine green, 0.5 g.
  { Water, 100 cc.

Mix a pipette full of A with a pipette full of B; stain two to eight minutes; dehydrate rapidly and mount in balsam.

Safranin.—

Safranin, 1 g.
95 per cent. alcohol, 50 cc.
Water, 50 cc.

Gentian Violet.—

Gentian violet, 1 g.
95 per cent. alcohol, 20 cc.
Water, 80 cc.
Anilin, 3 cc.

Orange G.—

Grange G, 1 g.
Water, 100 cc.

Bismark Brown.—

Bismark brown, 2 g.
70 per cent. alcohol, 100 cc.

Nigrosin.—

Nigrosin, 1 g.
Water, 100 cc.

Gram's Solution.—

Iodine, 1 g.
Iodide of potassium, 2 g.
Water, 300 cc.

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